XXIII. THE CRITICAL SOLUTION POINT OF URINE.

By WILLIAM RINGROSE GELSTON ATKINS and THOMAS ARTHUR WALLACE.

From Trinity College, Dublin.

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In order to determine the efficiency of a kidney as an excretory organ, the most exact method is the determination of the total solid content of the urine (neglecting colloids), as shown by the depression of its freezing-point below that of pure water. The examination of urine and blood by this method has proved very useful and reliable, but the time required for carrying it out constitutes a serious drawback when only one or two specimens are to be examined.

The following method was brought forward by one of us [Atkins, 1908] in the hope that it might be of service in cases where great accuracy is not essential and saving of time an advantage. Since no special apparatus is required and ice is not employed the usefulness of critical solution point determinations as a method of clinical examination is much increased especially for work in warm climates.

The present paper gives an outline of the method of such determinations, as the subject is a comparatively recent one. The data of the former paper are extended and the influence of the various constituents of the urine upon the critical solution point is traced out.

Outline of Critical Solution Point Theory.

(*a*) *Two pure substances.* Consider two liquids not completely miscible at room temperatures, such as phenol and water. If small quantities of these two substances are shaken together a white emulsion is obtained; this on standing separates into two layers, a lower one, consisting of phenol with some water dissolved in it, and an upper one of water with some dissolved phenol. If the temperature be now raised, the quantity of each dissolved in

the other increases till a temperature is reached at which the two become completely miscible; on cooling even a tenth of a degree separation again occurs, a white emulsion is formed, and on standing the two layers separate out as before. The temperature, at which the two layers become homogeneous, depends upon the proportions of water and phenol initially present, and rises in the case of water as the amount of phenol is increased until a mixture is reached which remains in the two phases at a higher temperature than any other mixture of the two pure components.

The composition of this mixture is termed the "critical concentration," and the temperature at which the liquid becomes homogeneous is called the "critical solution temperature." This temperature is accordingly that at which the two phases have the same composition and the same properties. At the critical concentration and critical solution temperature the volumes of the two phases, water in phenol and phenol in water, are equal.

At the critical temperature the two phases have the same surface tension, and are therefore completely miscible; at a very slightly lower temperature the surface tensions of the two phases are almost equal, consequently the meniscus between them is flat. As the homogeneous mixture cools, at a temperature very little above the critical temperature a beautiful opalescence appears, which is blue by reflected light and brown by transmitted.

If now a series of mixtures of water and phenol be taken and the temperatures at which they become homogeneous be determined, and the values thus obtained be plotted as ordinates, taking the corresponding percentage compositions of the mixtures as abscissae, a curve is obtained, the highest point of which is the critical solution point [Fig. 1, Schreinemakers, 1899]. This curve is somewhat flat at the top, hence the composition of the mixture may be slightly altered to one side or the other of the critical concentration without altering the temperature of miscibility more than about 0·1° C. The blue opalescence is still obtained, but it is not as beautiful as at the critical solution point itself.

(*b*) *Effect of a third substance.* The addition of a very small quantity of a third substance to any mixture of water and phenol raises the temperature at which the two phases become homogeneous, provided the added substance be freely soluble in water and almost or quite insoluble in phenol, or *vice versa*. If the added substance be freely soluble in both, then the temperature at which a homogeneous mixture is obtained is lowered. Thus by adding a small quantity of sodium oleate to a mixture of phenol and water, it is possible to obtain a very strong solution of phenol, homogeneous at room temperatures. Lysol, the disinfectant, is prepared in a similar way.

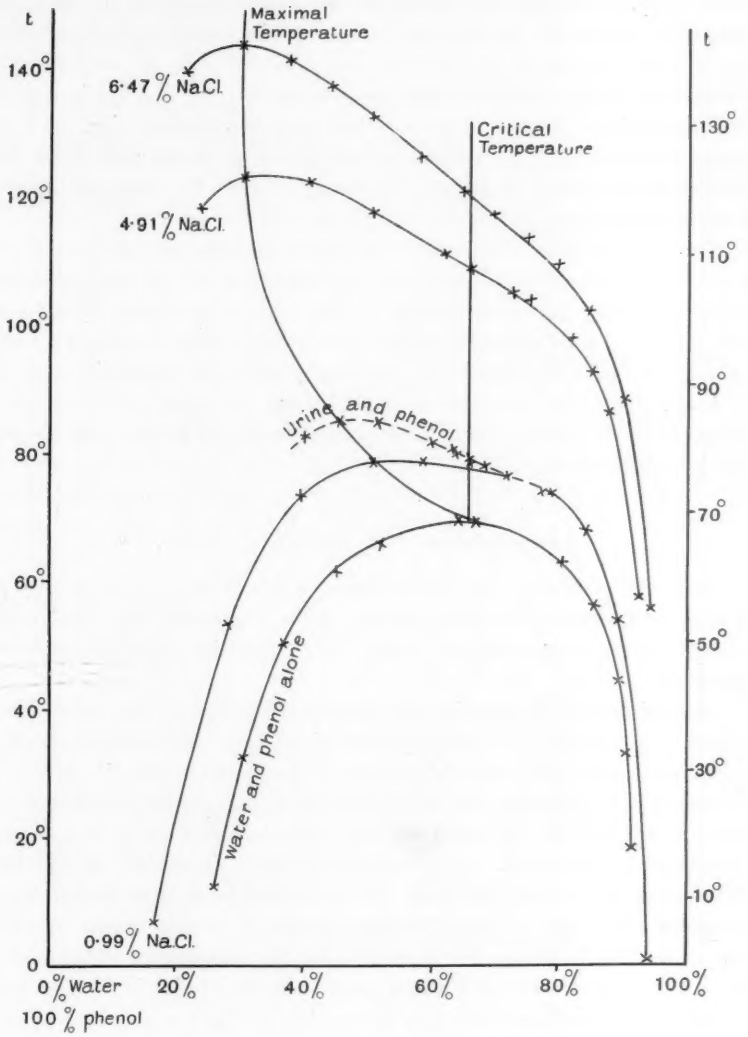

Fig. 1. In part after Schreinemakers. The dotted line shows the data for urine given in Table I.

In the case of a substance much more soluble in water than in phenol, if curves (Fig. 1) constructed as described be plotted when one of the pure liquids is replaced by a dilute solution of the third substance in that liquid, the general outline of the original curve is followed, the ordinates of temperature being everywhere greater and markedly so in what is termed the crest region. In each of these curves there is a critical point, but it no longer coincides with the maximum temperature as is the case when two pure components are concerned; accordingly it must be recognised by the beautiful opalescence and equality of volume of the layers.

The rise of critical temperature for dilute solutions, say one per cent., is roughly proportional to the number of gram-molecules of the substance added, with regard to its own solvent. This rise is very considerable, for an amount of solid sufficient to depress the freezing point of water 0·5° may raise the critical temperature of a mixture of water and phenol by as much as 4—5°. This rise therefore which can readily be determined to 0·1° gives about the same accuracy as a freezing point measured to 0·01° with greater facility and simple apparatus.

Application of the Method to Urine.

If in the preceding experiments urine, which is an aqueous solution of certain compounds, be substituted for water, a considerable rise in the critical solution temperature is found. This varies with the concentration of the urine.

A specimen of normal urine was examined throughout the whole curve, which was found to be similar to that given by a single substance when added to a water and phenol mixture, as shown by the broken line in Fig. 1. The method of procedure was as follows:—Weighed quantities of phenol and urine were mixed in test tubes, and the temperatures at which they became homogeneous were noted. A thermometer graduated in tenths from 50—100° is suitable, but for clinical work one graduated in degrees is sufficiently accurate. It is not so rough a measurement as it may appear to those accustomed to Beckmann thermometers, for the temperature differences to be measured are nearly ten times as great as those met with in freezing point work. The data from which the broken line in Fig. 1 was constructed are given below.

Inspection of the above table shows that the critical solution point of the phenol-urine mixture is at 79·7°, the critical concentration being 36 per cent. phenol. The critical solution point is by no means at the crest of the curve,

but is recognised by the characteristic opalescence. The critical solution temperature of the phenol-water mixture similarly determined is 66·5°. Thus the rise produced by the solids dissolved in the urine amounts to 13·2°.

TABLE I.

Percentage weight, phenol	Percentage weight, urine	Temperature of homogeneity	Notes
22·42	77·58	73·4	——
31·31	68·69	78·0	——
33·99	66·01	78·3	Opalescence.
34·28	65·72	78·7	Good opalescence.
35·93	64·07	79·7	Best opalescence.
36·48	63·52	80·0	Good opalescence.
39·78	60·22	81·2	No opalescence.
48·45	51·55	84·3	——
54·22	45·78	84·7	Maximum temperature = crest point.
60·06	39·94	82·2	

Having established the similarity between the curve afforded by urine and by a single added substance it is quite sufficient to determine the critical solution point of any urine with phenol in the following manner. Dry crystalline phenol is placed in a test tube to a depth of about 1 cm., and

TABLE II.

Normal Urine.

No.	Δ	R_c	$\frac{R_c}{\Delta}$
1	0·98°	7·7°	7·86
2	1·07	9·4	8·78
3	1·07	9·7	9·06
4	1·16	8·5	7·34
5	1·41	10·4	7·39
6	1·45	11·6	8·00
7	1·47	11·3	7·68
8	1·47	12·5	8·50
9	1·63	11·9	7·28
10	1·67	12·3	7·36
11	1·71	14·0	8·19
12	1·73	12·5	7·21
13	1·79	15·0	8·38
14	1·85	16·3	8·80
15	1·87	11·8	6·31
16	1·87	15·9	8·71
17	1·87	15·5	8·28
18	1·87	16·0	8·55
19	1·92	15·3	7·96
20	2·48	17·4	7·00
		Mean	7·93

slightly more than enough urine to cover it is added from a pipette. The mixture is warmed and, if the critical opalescence does not appear on cooling, urine is added in small quantities till a good opalescence is shown. In this manner one proceeds from a too concentrated mixture of phenol to one of the critical composition without the trouble of weighing the components. These determinations can be very rapidly made, the time depending on how nearly the critical concentration has been reached at the first trial.

A number of normal urines were examined by this method, their freezing points being also obtained in the usual manner, and opposite the depression Δ the rise in critical solution temperature R_c has been tabulated. A third column gives the ratio R_c/Δ, as shown in Table II.

It is clear from the above figures that there is a roughly constant ratio between the two sets of numbers. The fluctuations however are considerably beyond the range of any likely experimental error, though with highly coloured urines it is not easy to decide as to the most characteristic opalescence. (In practice it is permissible, within limits, to dilute such specimens, but in obtaining the above results this was avoided.) Thus in Nos. 15–18, which have the same freezing point, it is seen that in three cases the critical solution points are within 0·5°, whereas the fourth is widely divergent. As it seemed probable that such cases were to be explained by some appreciable differences in the relative proportions of the constituents of the urines in question, the most important of these were estimated in a number of specimens. Furthermore, in order to study the effect of one constituent alone, this was added in varying quantities to the analysed urine. Table III shows the analyses of Nos. 4, 5, 6, 10, 15 of Table II, expressed as grams per 100 cc.

TABLE III.

No.	Cl'	PO_4'''	SO_4''	$NH_4^{\cdot}$	Urea	Uric acid	Total N	Δ	R_c	$\frac{R_c}{\Delta}$
4	0·351	0·225	0·173	0·059	1·612	0·015	0·909	1·16	8·5	7·34
5	0·270	0·289	0·238	0·056	1·902	0·021	1·074	1·41	10·4	7·39
6	0·721	0·227	0·138	0·049	1·542	—	0·843	1·45	11·6	8·00
10	0·756	0·222	0·236	0·078	1·854	—	1·080	1·67	12·3	7·36
15	0·596	0·270	0·251	0·097	2·252	—	1·397	1·87	11·8	6·31
						Dextrose				
10*a*	0·453	0·133	0·141	0·047	1·113	2·02	0·648	1·21	10·0	8·26
10*b*	0·226	0·066	0·070	0·023	0·556	3·53	0·324	0·86	6·8	7·86

The analyses were made in the usual manner as described by Neubauer-Huppert, *Analyse des Harns*. The first urea estimations were made by the method of Benedict and Gephart [1908] while in the later ones Folin's new method was employed [1912]. The latter gives slightly lower results than the former, owing probably to the fact that in it uric acid is not hydrolysed.

In considering what effect the various constituents will have upon the critical solution point, it must be remembered that whereas the salts are far more soluble in water than in phenol, urea is soluble in both. It has been shown, moreover, that urea forms a compound with phenol; this, however, is very largely dissociated [J. C. Philip, 1903]. Accordingly, though an increase of urea causes an increase in the depression of freezing point, it causes a decrease in the critical solution point temperature as shown later on.

The analyses recorded show that Nos. 4, 5, 10 are very similar in composition, the only notable difference being the larger chloride content of No. 10. It may be seen that the ratio R_c/Δ is for these specimens quite constant. In No. 6, while the chloride percentage is high, that of urea is relatively low and R_c/Δ has increased; whereas, in No. 15, the diminished amount of chloride and the large rise in urea and total nitrogen combine to lower R_c/Δ very considerably.

To study the effect of increase in chloride, the sodium salt was added in various proportions to No. 4, as shown in Table IV.

TABLE IV.

Chloride alone varied.

No.	Cl′	Δ	R_c	$\frac{R_c}{\Delta}$
4	0·351	1·158°	8·5°	7·34
4*x*	0·703	1·472	12·8	8·70
4*y*	1·055	1·797	18·0	10·02
4*z*	2·111	2·780	29·7	10·69

Here the effect of the chloride in raising the critical solution point is well brought out by the increased values of R_c/Δ. Separate studies of the other salts were not made, as, owing to their solubility relations, their effects must be much the same as that of the chloride. Also they seem to vary less and occur in smaller quantities.

The addition of pure urea was next studied by adding weighed quantities to No. 5. This, of course, alters the amount of total nitrogen also.

TABLE V.

Urea and total nitrogen varied.

No.	Urea	Total nitrogen	Δ	R_c	$\frac{R_c}{\Delta}$
5	1·902	1·074	1·408°	10·4°	7·39
5*x*	2·446	1·328	1·527	10·2	6·68
5*y*	2·992	1·583	1·721	9·5	5·52
5*z*	4·628	2·345	2·157	7·8	3·62

Thus while urea alone lowers the critical solution temperature, its effect in urine is to lessen the rise produced by the saline bodies. Consequently a specimen which contains more urea than is usual, will have a smaller value for R_c/Δ. Thus the fluctuations in this ratio, as seen in Table II, are explained by the mutually counteracting effects of the salts and urea upon the critical solution point.

Pathological Urines.

It remains to consider to what extent pathological conditions of the urine may alter the constancy of the ratio R_c/Δ. The simplest case is, perhaps, that in which the urine is normal in its constituents, but dilute. Table VI shows the results obtained by examining both normal urines after dilution and pathological urines.

TABLE VI.

Normal diluted and pathological dilute urines.

No.	Nature of specimen	Δ	R_c	$\frac{R_c}{\Delta}$
21.	Normal diluted	0·33°	3·2°	9·70
22.	,, ,, ...	0·44	4·7	10·70
23.	,, ,, ...	0·87	7·6	8·74
24.	Pathological	0·70	7·0	10·00
25.	From right kidney of *A*, by catheterisation ...	0·47	4·5	9·58
26.	From left kidney of A, ,, ,, ...	0·73	7·0	9·58
27.	From both kidneys of B, morning; nothing but water since previous evening	0·63	5·5	8·73
28.	From right kidney of *B*, by catheterisation at 1 p.m. Conditions as above. Albumin present ...	0·24	2·5	10·42
29.	From left kidney of *B*, by catheterisation. Conditions as above. No albumin	0·30	3·0	10·00
			Mean	9·72

These figures show that in very dilute urines the salts have a somewhat greater effect in raising the critical solution temperature than they have in those of average concentration. It is, however, to be noted that with such small values of R_c, an error of 0·1° affects the value of R_c/Δ very considerably. The similarity of R_c/Δ in Nos. 25 and 26, and also in Nos. 28 and 29, is noteworthy, and gives reason for supposing that the two kidneys were in each case excreting fluids almost identical in composition though not in concentration. The identity in composition of the urine from the right and left kidney is now completely established as a normal occurrence, though the earlier work seemed to point to the opposite conclusion [Casper and Richter].

A small number of other pathological urines were examined, as shown in Table VII.

TABLE VII.

No.	Nature of specimen	Δ	R_c	$\frac{R_c}{\Delta}$
30.	Chronic parenchymatous nephritis, albumin present ...	1·12°	9·3°	8·30
31.	Specimen with albumin, which was removed by boiling	1·04	9·3	8·94
32.	Alkaline, much albumin	1·27	11·0	8·66

In the above three instances, high values of R_c/Δ were obtained, probably due to a preponderance of salts in the urines. Albumin, being a colloid, can hardly be expected to have any perceptible effect either in raising or lowering the critical solution temperature. In the presence of the phenol in the critical concentration (36 %), the albumin in any specimens met with was not precipitated, even by boiling for five minutes.

Diabetic Urines.

As the presence of dextrose has a great effect upon the freezing point it was thought well to see what alteration in the critical solution temperature its presence in urine would cause. As it is somewhat soluble in phenol, its addition to a phenol-water mixture was first studied. Results are shown in Table VIII.

TABLE VIII.

Dextrose added to phenol-water system.

Dextrose, grams per 100 cc. water	Δ	R_c	$\frac{R_c}{\Delta}$
5·05	0·56°	1·7	3·04
4·04	0·42	1·0	2·38
2·02	0·21	0·4	1·90
1·01	0·10	0·3	3·00
0·50	0·05	−0·1	−2·00

It will be seen that the addition of dextrose causes a relatively small rise in the critical solution temperature, the last figure being obviously due to experimental error. As before pointed out, with low values of R_c small errors cause the ratio R_c/Δ to fluctuate greatly. The effect of dextrose in a urine will, therefore, be to lower the value of R_c/Δ, for though it causes a rise in R_c yet this is not so great as is the case with the salts. To test this point further, No. 10 was diluted by the addition of dextrose solution, so as to have the

composition recorded in Nos. 10 *a* and 10 *b*, in Table III. Also dextrose was weighed out and made up to 100 cc. with No. 15, so that the resulting solutions had very closely the composition of No. 15, but not exactly on account of the volume occupied by the dissolved dextrose. The figures obtained are shown in Table III and in Table IX.

TABLE IX.

Dextrose added to phenol-urine system.

No.	Dextrose, grams per 100 cc. urine	Δ	R_c	$\frac{R_c}{\Delta}$
10	—	1·67°	12·3°	7·36
10 *a*	2·02 (No. 10 diluted)	1·21	10·0	8·26
10 *b*	3·53 (No. 10 diluted)	0·86	6·8	7·86
15	—	1·87	11·8	6·31
15 *a*	1·08	1·98	11·3	5·71
15 *b*	5·40	2·43	12·3	5·06

Here it is seen that in 10 *a* and 10 *b* the dilution effect has more than counterbalanced the addition of dextrose, so that R_c/Δ is slightly raised. In 15 *a* and 15 *b*, however, it is evident that the already low value of R_c/Δ afforded by No. 15 is depressed still further.

In Table X are shown the values given by diabetic urines, not intentionally altered in any way. They had not been heated.

TABLE X.

Diabetic urines.

No.	Dextrose, grams per 100 cc.	Δ	R_c	$\frac{R_c}{\Delta}$
33	1·87	1·17°	8·0°	6·84
34	5·62	1·56	10·0	6·41
35	?	1·75	11·0	6·30
36	0·19	1·94	15·3	7·88
37	2·34	2·03	16·0	7·88
38	3·38	2·27	16·5	7·56
			Mean	7·14

Here again the mean value of R_c/Δ is lower than with normal urines, though, owing to the variations of the other constituents of the urine, the decrease is not proportional to the sugar concentration.

We hope to make further experiments with pathological urines, taking into account other abnormal constituents of diabetic specimens.

Summary and Discussion of Results.

The examination of a considerable number of normal urines has shown that the rise in critical solution temperature is roughly eight times as great as the depression of freezing point. This holds good for urines of normal concentrations, but with very dilute urines the critical solution temperature rise is somewhat greater, consequently R_c/Δ has a value greater than eight. This is also the case in specimens which, for any cause, contain an unusually large quantity of salts. On the other hand, the presence of an exceptionally great amount of urea occasions a smaller rise of critical solution temperature; glucose, when present in quantity, has the same effect, but it is far less marked. In the last two cases accordingly the ratio R_c/Δ is somewhat below eight.

This relation between the freezing point depression and the rise in critical solution temperature enables one to transfer the conclusions established by the former method to the latter. Thus A. v. Koranyi [1897, 1899: Quoted from Casper and Richter] gives $1{\cdot}3° - 2{\cdot}3°$ as the usual limits for the freezing point of urine, from a normal kidney under ordinary conditions. Taking $R_c/\Delta = 8$, this range corresponds to a rise in critical solution temperature of $10{\cdot}4° - 18{\cdot}4°$.

Lindemann [1899: Quoted from Casper and Richter] gives a more extended range, $0{\cdot}9° - 2{\cdot}7°$, for values of Δ, and states that kidney patients almost always excrete urine for which Δ is less than $1{\cdot}0°$. Accordingly, taking Lindemann's limit, $0{\cdot}9°$, and allowing for experimental error and fluctuations due to the composition of the sample, it seems safe to take a rise of 8° C. in the critical solution temperature as indicating that the kidney supplying the sample was functioning satisfactorily. It must be remembered, however, that the converse is not always true; thus a specimen giving a rise of 7° might really be normal, as, owing to the ingestion of a large amount of liquid, or to the presence of much urea, the value of R_c could very well be lowered.

In the latter limiting case, it would be well to make a freezing point determination or rough urea determination by the hypobromite method, to see if there was an unusual quantity of this substance. Ordinary values of R_c lie between 11° and 16° C.

In conclusion, we wish to thank Dr J. Timmermans, of Brussels, for putting his extensive knowledge of critical solution point determinations

entirely at our disposal during his visit to Trinity College. A detailed treatment of the subject will be found in his paper [1907][1].

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[1] See also Findlay, *The Phase Rule*, and Young, *Stoichiometry*. London, Longmans, Green & Co.

XXIV. QUANTITATIVE RELATIONS IN CAPILLARY ANALYSIS.

By HANS SCHMIDT.

From the Bacteriological Department, Lister Institute.

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When a dispersed system is allowed to fall on filter- or blotting-paper or to rise up into a strip of such a paper, one observes that the dispersed phase remains behind the medium of dispersion, which is in most cases water. This phenomenon, which involves an increase in the concentration of the dispersed phase in the paper, can be used for the qualitative testing of the nature of the substance as has been shown by the capillary analytical experiments of Goppelsroeder [1909].

Holmgren [1908] tried to establish a formula, which gives a quantitative relation between the extents of the dispersion medium and the dispersed phase. He experimented with diluted acids, especially with hydrochloric acid, by allowing the latter to drop on blotting-paper. Using Congo-red as indicator, he was able to show how far the acid was spread over the paper compared with the water. If a good and homogeneous blotting-paper is used, the figure caused by the diffusion of the drop approaches a circle.

Holmgren supposed that the relation between the water-zone, the largest radius of which may be R, and the inner circle of radius r caused by the acid, is constant for each concentration, so that the quotient of two different concentrations is equal to the quotient of these relations. Therefore, as $[\pi R^2 - \pi r^2]$ is the surface of the water-ring, the following relation exists according to Holmgren:

$$\frac{C}{C_1} = \frac{\frac{R^2 - r^2}{r^2}}{\frac{R_1^2 - r_1^2}{r_1^2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1).$$

If we signify by P that concentration for which

$$\frac{r_1^2}{R_1^2 - r_1^2} = 1,$$

the following equation results:

$$C = P \cdot \frac{r^2}{R^2 - r^2} \quad \ldots\ldots(2),$$

or

$$P = C \cdot \frac{R^2 - r^2}{r^2} \quad \ldots\ldots(3).$$

If, therefore, the concentration C and the radii of the circles produced on blotting-paper by any drop of the acid are known, P is determined from equation (3). Holmgren supposed P to be constant for the same paper and independent of the concentration. He called P the paper constant and was then able to determine any other concentration by the equation (2), but with the limitation that this method was not to be used except for very dilute solutions between 2·0 and 0·01 %. The results of Goppelsroeder's experiments, which give only the measurements of capillary height in strips of filter-paper, can also be treated by this formula of Holmgren, if instead of the height-measurements their squares are taken.

Skraup [1909, 1 and 2] and his co-workers were able to confirm the applicability of this formula for capillary-analysis. On examining the amount of free hydrochloric acid in the gastric juice by this method, I found [Schmidt, 1913] that the results obtained agreed very closely with those obtained by titration. Later some doubts arose in my mind as to the correctness of Holmgren's formula and occasioned me to undertake the following investigation.

Technique of Experiments.

I experimented exclusively with hydrochloric acid, an exactly adjusted normal solution of which was taken as the basis for the following dilutions between 2·0 and 0·05 %. One drop of these solutions was allowed to fall on blotting-paper from a short distance. Much attention was paid to insure that the drop had always the same volume. With an ordinary pipette it was not possible to get the volumes of the drops as exact as was desirable. I therefore used the dropping surface of Traube's stalagmometer, which gives exactly equal drops, if the temperature be kept constant and the instrument be not allowed to deviate from the vertical position. Blotting-papers are much better fitted for capillary analysis than filter-paper, but they must not be too thick and must have a smooth surface and a most homogeneous structure. I obtained a series of different blotting-papers from the firm of C. Schleicher und Schüll, Düren i. Rheinland. The papers marked Nos. 123

and 117 proved to be specially suitable for my purpose. The papers were stained by floating them rapidly through an alcoholic solution of Congo-red (0·04 %) or methyl-orange (0·1 %). In order to prevent the water from evaporating or at least to reduce the evaporation to a minimum, as soon as the drop was absorbed the paper was placed between two glass plates and a weight laid on the plate to avoid any wrinkling. The plates were then put on a small frame under a glass globe, the interior of which was kept saturated with moisture by means of wetted filter-paper below. To neglect the evaporation leads to false conclusions. The radii were measured in millimetres by the use of transmitted or reflected light, and since the figures of the larger spots are mostly elliptical owing to the direction of the fibres in the structure of the paper, the main axes as well as two oblique axes were measured, so that the given result is the average of the measurement of 4 diameters. The number of figures examined to determine any single point is in most cases at least 4. The measurement took place after exactly one hour, the process having then practically reached a standstill. In reality one may sometimes observe an increase of the radii even after 24 hours, but the amount of it is too insignificant to be noted, especially if only one drop is taken.

Referring to dilutions of acid under 0·1 % the measurement must take place earlier than one hour especially when Congo-red is used, because the blue colour disappears after a certain time, owing to chemical action.

Experiments.

The mass of one drop given by a stalagmometer at the temperature of 18–20° is indicated by a. If the drop consisted of distilled water, its weight was found to be 0·1160 gr., and if it consisted of HCl of a concentration between 2·0 and 0·05 %, I found the average weight of a drop 0·116 gr., the differences of the specific gravity in so highly diluted acids being so small that they can be neglected without introducing an error which exceeds the error of observation.

Therefore, putting the volume equal to the weight (in case of dilute HCl) I give a the value of 0·116 gr. Supposing m parts of hydrochloric acid to be present in the drop a, the concentration is given by $C = \frac{m}{a}$ by means of which m is easily calculated.

Under the above described conditions, a series of observations was made, of which the following may be reproduced:

TABLE I.

Paper No. 123. $a = 0{\cdot}116$ gr.

Indicator:		Congo-red						Methyl-orange			
Number of drops:—		1 a		2 a		3 a		1 a		2 a	
HCl %	m	R	r	R	r	R	r	R	r	R	r
2·0	2·32	23·12	21·87	32	29·37	37·87	34·25	23·12	21·62	29·81	26·43
1·0	1·16	23·5	19·62	32·37	26·5	38·5	31·25	23·37	19·56	31·75	26
0·75	0·87	24	18·37	32·37	24·5	38·7	29·5	23·37	18	32	24·25
0·5	0·58	23·62	16·56	31·87	22·18	37·8	26·5	24	16·87	32·25	22·43
0·4	0·464	23·87	15·75	32·75	21·5	38·2	22·12 (?)	23·62	15·56	32·37	20·75
0·3	0·348	23·62	14·63	32·25	19·68	37·0	23·5	23·62	14·75	31·62	19·56
0·2	0·232	23·37	12·37	31·5	17·12	37·3	20·62	23·62	12·25	32·12	17·12
0·1	0·116	23·5	10	31·62	13·5	37·8	16·62	23·62	10·75	32	13·75
0·05	0·058	23·7	8·62	—	—	—	—	23·43	—	—	—
Means of R		23·48		32·09				23·53		31·74	

TABLE II.

Paper No. 117. $a = 0{\cdot}116$ gr. Indicator: Congo-red.

Number of drops:		1 a		2 a		3 a	
HCl %	m	R	r	R	r	R	r
1·0	1·16	26·5	24·25	36·0	32·41	42·25	37·5
0·75	0·87	26·85	22·40	35·75	30·1	42·5	35·5
0·5	0·58	26·68	20·62	35·43	27·75	41·62	33·18
0·4	0·464	26·87	20·5	35·43	27·5	43	33·0
0·3	0·348	25·87	18·85	35·87	25·2	42·37	30·3
0·2	0·232	26·25	16·37	36·0	22·81	42·0	26·75
0·1	0·116	26·25	13·63	35·75	18·5	42·25	22·75
0·05	0·058	26·75	—	—	—	—	—
Means of R		26·50		35·75		42·28	

TABLE III.

Paper No. 123. $a = 0{\cdot}0311$ gr.

Indicator:		Congo-red		Methyl-orange	
HCl %	m	R	r	R	r
1·0	0·311	14·75	13·29	—	—
0·5	0·155	15·1	12·5	12·5	9·3
0·4	0·124	14·65	9·4	12·8	8·9
0·3	0·0933	14·84	8·85	12·7	8·25
0·2	0·0622	15·15	7·95	12·8	7
0·182	0·0566	13·3	6·4	12·9	6·36
0·1	0·0311	14·26	6·0	13·1	6
Means of R		14·6		12·8	

On the assumption that the aggregation of acid in the inner circle is of the nature of an adsorption process, the relationship between the two variables m and r must be expressible by the exponential equation

$$r = \beta m^{p}.$$

It is highly probable also that the concentration of the acid or that of the hydrogen ions is continuously decreasing from the centre to the periphery of the spot-system in the case of any drop of a certain concentration falling on the paper. I suppose then, that r indicates that point of hydrogen ion concentration which corresponds with the sensibility of the indicator. But owing to the impossibility of proving experimentally the gradual decrease of the hydrogen ion concentration in the paper between the centre and r, my supposition remains an hypothesis. To show how in different concentrations the relation between m and r is expressible by the equation $r = \beta m^p$, we use the graphic method in constructing the m-r-curve Fig. 1.

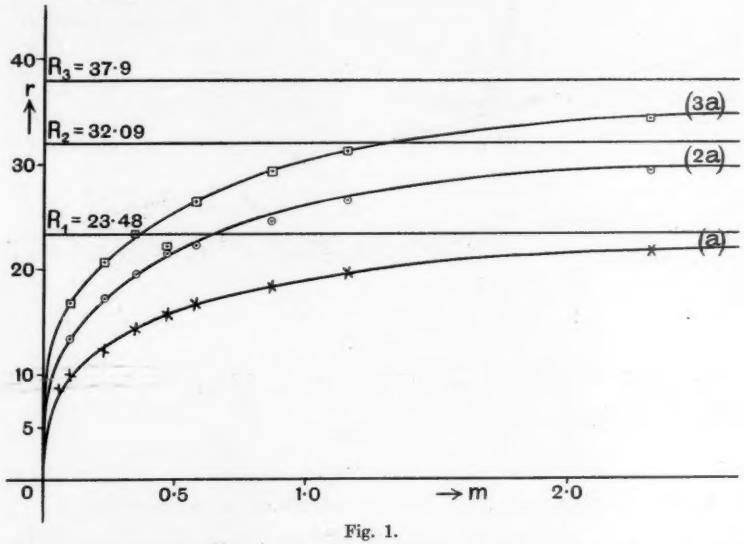

Fig. 1.

The m-r-curve rises very steeply in the beginning and then runs nearly parallel to the m-axis, asymptotically approaching a straight line, of which the equation is $r = R$. The difference $(R - r)$ is identical with the breadth of the water-zone and the latter is of course equal to R in case of distilled water, when m or $C = 0$. $(R - r)$ decreases with increase in the amount of m. Theoretically, the decrease of $(R - r)$ is infinite but in reality m reaches very soon such an amount, that the water-zone is no longer visible. When the latter effect occurs with one drop, the phenomenon can still be obtained by increasing the volume of the drop or augmenting the number of drops, but the errors of observation are also increasing and in such a

degree that for concentrations higher than 2·0 % the method of Goppelsroeder is preferable, which, as already mentioned, involves the measurement of the capillary height in strips of filter-paper.

That R is in reality the constant, which it seems to be in the experimental data, and that R depends—in the same paper—only upon the volume of the drop and not upon the concentration, will be shown later.

By taking the logs. of the equation $r = \beta \,.\, m^p$, we get

$$\log r = p \,.\, \log m + \log \beta.$$

This is the equation of a straight line, which is very nearly approached by plotting the values of $\log r$ and $\log m$, as is shown in Fig. 2.

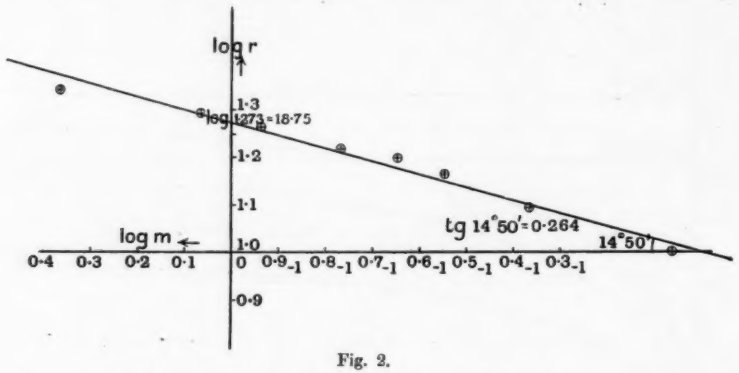

Fig. 2.

By graphic interpolation according to Freundlich's [1907] procedure the values of β and p are found to be

$$p = 0{\cdot}264$$

and

$$\beta = 18{\cdot}75.$$

In the following table the values are calculated by the equation

$$r = 18{\cdot}75 m^{0{\cdot}264} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4).$$

TABLE IV.

Paper 123. Congo-red. 1 drop ($a = 0{\cdot}116$ gr.), $r = 18{\cdot}75 m^{0{\cdot}264}$.

C = % HCl	m	r calculated	r observed	Δ calc. – obs.
2·0	2·32	23·41	21·87	– 1·54
1·0	1·16	19·49	19·62	+ 0·13
0·75	0·87	18·08	18·37	+ 0·29
0·5	0·58	16·31	16·56	+ 0·15
0·4	0·464	15·12	15·75	+ 0·63
0·3	0·348	14·19	14·63	+ 0·44
0·2	0·232	12·74	12·37	– 0·37
0·1	0·116	10·61	10	– 0·61
0·05	0·058	8·84	8·62	– 0·22

Although the agreement is fairly good, a further calculation of β and p was made by the following procedure in order to diminish the differences.

HCl %	$100 \times m$	$100 \times r$	$\log m$	$\log r$
2·0	232	2187	2·36549	3·33985
1·0	116	1962	2·06446	3·29248
0·75	87	1837	1·93952	3·26411
0·5	58	1656	1·77085	3·21906
0·4	46·4	1575	1·64652	3·19728
0·3	34·8	1463	1·54158	3·16524
0·2	23·2	1237	1·36549	3·09237
0·1	11·6	1000	1·06446	3
0·05	5·8	862	0·76343	2·93551
		Mean	1·613533	3·167322

Deviations from means:

m_1	r_1	$m_1 . r_1$	$(m_1)^2$
+0·75195	+0·172528	0·12957	0·56543
+0·45092	+0·125158	0·056432	0·20333
+0·32598	+0·096788	0·031551	0·051067
+0·15731	+0·051738	0·0081424	0·024746
+0·032987	+0·029958	0·00098825	0·0010881
−0·071953	−0·002082	0·000149806	0·0051772
−0·248043	−0·054952	0·013630	0·061523
−0·549073	−0·167322	0·091871	0·30147
−0·850103	−0·231812	0·19705	0·72267

$$P = \frac{\Sigma (m_1 . r_1)}{\Sigma (m_1)^2} = \frac{0{\cdot}529384}{1{\cdot}9365013} = 0{\cdot}2733.$$

The value 0·273 for the exponent p gives the following values for

$$\log \beta_1 = \log r - 0{\cdot}273 \log m.$$

$\log \beta_1$	and for β_1
2·694072	494·4
2·728883	535·6
2·73462	542·7
2·73561	544·0
2·74778	559·4
2·74438	555·1
2·711959	524·3
2·70940	512·1
2·72709	533·4

As the figures for r and m have been multiplied by 100, the values of $\log \beta$ in the equation

$$r = \beta . m^p$$

are calculated from the following equation:

$$\log \beta = \log \beta_1 + 2 . (0{\cdot}273) - 2.$$

These values of $\log \beta$ are therefore the following:

$\log \beta$	β
1·25007	17·78
1·27488	18·83
1·28062	19·08
1·28161	19·12
1·29378	19·66
1·29038	19·51
1·26559	18·43
1·25540	18·00
1·27309	18·75
	Mean of β 18·88

It results therefore for the relation between r and m the equation

$$r = 18{\cdot}88 \,.\, m^{0{\cdot}273} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5).$$

Thus substituting the calculated values for β and p the following equation was obtained:

$$r = 18{\cdot}88 \,.\, m^{0{\cdot}273},$$

which gives a far better agreement between calculation and observation, as is shown in the following table:

TABLE V.

Paper 123. Indicator: Congo-red. One drop. $a = 0{\cdot}116$ gr. $r = 18{\cdot}88 \,.\, m^{0{\cdot}273}$.

$C = \%$ HCl	m	r calculated	r observed	Δ
2·0	2·32	23·75	21·87	−1·88
1·0	1·16	19·66	19·62	−0·04
0·75	0·87	18·17	18·37	+0·2
0·5	0·58	16·34	16·56	+0·22
0·4	0·464	15·11	15·75	+0·64
0·3	0·348	14·15	14·63	+0·48
0·2	0·232	12·67	12·37	−0·3
0·1	0·116	10·48	10·0	−0·48
0·05	0·058	8·677	8·62	−0·05
		Idem, but $a = 0{\cdot}0311$ gr.		
1·0	0·311	13·72	13·29	−0·43
0·5	0·155	11·09	12·5	+1·41
0·4	0·124	10·67	9·4	−1·27
0·3	0·0933	9·880	8·85	−1·03
0·2	0·0622	8·845	7·95	−0·89
0·182	0·0566	8·620	6·4	−2·42
0·1	0·0311	7·320	6	−1·32

The reason why the second part of the Table V does not show so good an agreement is the inexact measurement of drops by using an ordinary pipette instead of a stalagmometer. By increasing the number of drops, so that instead of a, $2a$ or $3a$ were taken of the same concentration $C = \frac{m}{a}$, the radii of the corresponding spots must be

$$r\sqrt{2}, \quad r\sqrt{3},$$

or, generally speaking, if $[na]$ is taken as the dropping mass, the corresponding

radius is $r\sqrt{n}$, r being the radius for a. Therefore, if the radii found for a are supposed to be fixed, the radii for $2a$ or $3a$ can be calculated. The following table gives the results:

TABLE VI.

Paper 123. Indicator: Congo-red. $a = 0{\cdot}116$ gr.

Number of drops:	a	$2a$		$3a$	
$C = \%$ HCl	r_1	r_2 calculated	r_2 observed	r_3 calculated	r_3 observed
2·0	21·87	30·83	29·375	37·63	34·25
1·0	19·62	27·66	26·5	33·98	31·25
0·75	18·37	25·90	24·5	31·81	29·5
0·5	16·56	23·34	22·185	28·68	26·5
0·4	15·75	22·20	21·5	27·27	22·125 (?)
0·3	14·63	20·63	19·685	25·33	23·5
0·2	12·37	17·44	17·125	21·42	20·625
0·1	10·0	14·14	13·5	17·32	16·625
0·05	8·62	—	—	—	—

The agreement between the calculated and observed values for r is still fairly good for $2a$. But by increasing the number of drops beyond 2, the experimental error increases, because the figure produced on paper loses the resemblance to a circle, so that the average of r is determined with more arbitrariness. By using the equation (5)

$$r = 18{\cdot}88 \,.\, m^{0{\cdot}273}$$

for cases where, generally speaking, (na) was taken, m becomes (nm). The values of r_2 for $2m$ and of r_3 for $3m$ are shown in the following table:

TABLE VII.

Paper No. 123. Indicator: Congo-red. $a = 0{\cdot}116$ gr. $r\sqrt{n} = 18{\cdot}88\,(nm)^{0{\cdot}273}$.

$C = \%$ HCl	$2m$	log $2m$	r_2 calculated	r_2 observed	Δ observed − calc.	Δ r_2 calculated $- r_2 = r_1\sqrt{2}$
2·0	4·64	0·66652	28·71	29·37	+0·66	−2·12
1·0	2·32	0·36549	23·75	26·5	+3·25	−3·91
0·75	1·74	0·24055	21·96	24·5	+2·54	−3·94
0·5	1·16	0·06446	19·66	22·18	+2·52	−3·68
0·4	0·928	0·96755 − 1	18·38	21·5	+3·12	−3·82
0·3	0·696	0·84261 − 1	17·10	19·68	+2·58	−3·53
0·2	0·464	0·66652 − 1	15·31	17·12	+1·81	−2·13
0·1	0·232	0·36549 − 1	12·67	13·5	+0·83	−1·47

$C = \%$ HCl	$3m$	log $3m$	r_3 calculated	r_3 observed	Δ observed − calc.	Δ r_3 calculated $- r_3 = r_1\sqrt{3}$
2·0	6·96	0·84261	32·06	34·25	+2·09	−5·57
1·0	3·48	0·54158	26·53	31·25	+4·72	−7·45
0·75	2·61	0·41664	24·53	29·5	+4·97	−7·28
0·5	1·74	0·24055	21·96	26·5	+4·54	−6·72
0·4	1·392	0·13364	20·53	22·12 (?)	+1·59 (?)	−6·74
0·3	1·044	0·01870	19·10	23·5	+4·4	−6·23
0·2	0·696	0·84261 − 1	17·10	20·62	+3·52	−4·32
0·1	0·348	0·54158 − 1	14·15	16·62	+2·47	−3·17

The deviations of the calculated figures of r from those obtained by observation are so considerable as to make it clear that the volume used for capillary-analytic purposes must not exceed a certain quantity. But the deviation of the calculated values $r_1\sqrt{n}$ and $18{\cdot}88m^{0{\cdot}273}$ must be due to the inexactness of the constant in the equation $r = \beta m^p$ and to the experimental errors for r_1. In reality the relation

$$r_1\sqrt{n} = \beta\,(n\,.\,m^p) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6)$$

must exist.

Theoretical Part.

On detailed consideration of the processes taking place in the paper, it becomes evident that an increase of concentration of the hydrochloric acid has taken place.

If C signify the original concentration and C_1 the resulting concentration in the coloured spot, the relation between these concentrations may be deduced in the following way. The paper is supposed to be previously stained either with 0·04 % alcoholic Congo-red solution, or with 0·1 % alcoholic methyl-orange solution, and the staining to be done in the same manner, the paper not being allowed to adsorb much of the dye, a process which is at room temperature only a matter of time, as has been shown by W. M. Bayliss [1906]. Observation shows that stained paper differs slightly in quality from unstained, a fact to which reference will be made later.

Let $[a]$ be the constant quantity of the drop and $[na]$ the quantity of hydrochloric acid of the concentration $C = \frac{m}{a}$, dropping on a piece of blotting-paper which is supposed to be always of the same quality. The quantity $[na]$ of the acid may spread over the paper, so that the acid reaches from the centre to the distance $r\sqrt{n}$, and the largest radius of the water-zone may be $R\sqrt{n}$, if r and R are the corresponding values for $[a]$. In case of another drop-volume, the weight being g', the radius becomes $r\sqrt{\frac{g'}{g}}$, g being 0·116 gr.

As the acid constituent of $[na]$ is in the interstices of the paper, the volume $[na]$ must be equal to the volume of the paper, the air being included, minus the specific volume of the paper-material itself in the same space. The volume occupied by $[na]$ is $\pi n R^2$ and the specific volume of the paper-material is its weight divided by its specific gravity s. The weight of a portion of paper of square millimetre surface and thickness δ is indicated by p. Therefore the following equation results for $[na]$:

$$na = \pi n R^2\delta - \pi n R^2\frac{p}{s} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7),$$

or, if $n = 1$,

$$a = R^2 . \pi \left(\delta - \frac{p}{s}\right).$$

$\pi \left(\delta - \frac{p}{s}\right)$ represents a constant characteristic for the paper and may be indicated by k, so that

$$k = \pi . \left(\delta - \frac{p}{s}\right)$$

and

$$a = R^2 . k,$$

or

$$R = \sqrt{\frac{a}{k}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (8).$$

That is to say, R depends only upon the variation in quality of the paper and upon the drop-volume which is taken, but it is independent of the concentration C. Therefore R is to be considered as constant for all concentrations, if the same paper and the same drop-volume are used.

The easiest and quickest way to find k is by means of the equation (8), but by weighing the paper and determining its specific gravity and thickness the same value for k will be obtained by the equation $k = \pi . \left(\delta - \frac{p}{s}\right)$.

In order to determine the thickness δ of the paper, a certain number of sheets are placed between two glass plates and by pressing them very tightly the measure of the distance may be found, which gives when divided by the number of sheets a rough value of δ. The best manner to determine the specific gravities is the use of a pyknometer. The following data were thus obtained:

Paper No. 123 $\delta = 0{\cdot}17$ mm.
$p = 0{\cdot}1408$ mgr.
$s = 1{\cdot}36$
$k = 0{\cdot}20923$

Paper No. 117 $\delta = 0{\cdot}16$ mm.
$p = 0{\cdot}118$ mgr.
$s = 1{\cdot}1$
$k = 0{\cdot}1651$

When the value of k is found in this way, R can be calculated by the equation (8) as is shown in the following table:

TABLE VIII.

		R calculated			R observed		
Paper No.	a (mgr.)	a	$2a$	$3a$	a	$2a$	$3a$
123	116	23·537	33·28	40·77	23·48	32·09	37·9
117	116	26·49	37·48	45·90	26·5	35·75	42·28
123	13·1	12·165	—	—	12·8 Methyl-orange 14·6 Congo-red		—

The agreement is very close for a, but here, too, it is apparent that increase of the number of drops increases the inexactness of the observations. If the value of R_1 is fixed, R_2 and R_3 can be calculated as $R_1\sqrt{2}$ and $R_1\sqrt{3}$, which give also a fairly good agreement, as the following figures demonstrate:

TABLE IX.

Paper No.	a (mgr.)	R_1	$R_2=R_1\sqrt{2}$	$R_3=R_1\sqrt{3}$
123	116	23·5	33·13	40·70
117	116	26·5	37·36	45·89

If $C=\frac{m}{a}$, the quantity na of HCl contains nm parts of it. These nm parts of HCl have been retained by adsorption in the inner circle (r), and if v indicates the volume of its interstices, the following equation must express the resulting concentration C_1:

$$C_1=\frac{n\,.\,m}{v}.$$

I neglect the possibility, that the fibres may swell, supposing that the adsorption process is much sooner finished than the swelling of the fibres.

By analogy with equation (7), v can be expressed by

$$v=\pi n r^2\delta-\pi n r^2\,.\,\frac{p}{s}.$$

After substituting this value for v, C_1 is given by

$$C_1=\frac{n\,.\,m}{n r^2\pi\,.\left(\delta-\frac{p}{s}\right)},$$

or

$$C_1=\frac{m}{r^2k} \qquad \text{.....(9).}$$

This is the equation giving the resulting concentration in the coloured spot, and if z represents the increase of concentration, so that

$$C_1=z+C,$$

and one substitutes

$$C_1=\frac{m}{r^2\,.\,k}$$

and

$$C=\frac{m}{a},$$

the following equation results:

$$\frac{m}{r^2k}=z+\frac{m}{a},$$

as

$$a=k\,.\,R^2,$$

$$z=\frac{m}{r^2k}-\frac{m}{R^2k},$$

$$z=\frac{mR^2-mr^2}{k\,.\,R^2\,.\,r^2}.$$

If we substitute $k \cdot R^2 = a$,

and $\frac{m}{a} = C$,

we find $z = C \cdot \frac{R^2 - r^2}{r^2}$ .....(10).

Therefore z is found to be equal to the value of the paper-constant P of Holmgren (3).

Holmgren was of opinion that the relation between the resulting concentration C_1 in the paper and the original concentration C can be expressed by the quotient $\frac{R^2}{r^2}$, so that

$$C_1 = \frac{R^2 C}{r^2} \quad \text{.....(11).}$$

This view seems to be correct, for putting

$$C_1 = \frac{R^2 \cdot C}{r^2} = \frac{m}{r^2 k},$$

we find $R^2 \cdot C \cdot k = m$, and substituting $R^2 k = a$, the definition of $C = \frac{m}{a}$ results.

When Holmgren eliminated $r^2 = \frac{C \cdot R^2}{P + C}$ out of the equation (3) and substituted this value for r^2 in the equation (11), he got

$$C_1 = P + C \quad \text{.....(12).}$$

Therefore Holmgren's paper-constant corresponds with z in equation (10) representing the increase of concentration by the adsorption, P therefore not being a constant but a function of the concentration in question. The following table shows the values for C_1 calculated by the equation (9), P calculated by the equation (2) and C_1 calculated by formula (12).

TABLE X.

Paper No. 123. Congo-red. One drop ($a = 0{\cdot}116$ gr.). $k = 0{\cdot}20923$.

$C = \%$ HCl	$C_1 = \frac{m}{r^2 \cdot k}$; %	$P = C\frac{R^2 - r^2}{r^2}$	$C_1 = P + C$	$P = 0{\cdot}441$ $C_1 = 0{\cdot}441 + C$
2·0	2·32	0·305	2·305	2·441
1·0	1·44	0·432	1·432	1·44
0·75	1·23	0·475	1·225	1·19
0·5	0·994	0·505	1·005	0·94
0·4	0·894	0·488	0·888	0·841
0·3	0·777	0·472	0·772	0·741
0·2	0·724	0·520	0·720	0·641
0·1	0·554	0·451	0·551	0·541
0·05	0·373	0·321	0·371	0·491

Mean of $P = 0{\cdot}441$.

TABLE X (*cont.*)

Paper No. 117. Congo-red. One drop ($a = 0{\cdot}116$ gr.). $k = 0{\cdot}1651$.

$C = \%$ HCl	$C_1 = \frac{m}{r^2 . k}$ (%)	$P = C\frac{R^2 - r^2}{r^2}$	$C_1 = P + C$	$P = 0{\cdot}285$ $C_1 = 0{\cdot}285 + C$
1·0	1·19	0·194	1·194	1·285
0·75	1·05	0·299	1·049	1·035
0·5	0·825	0·325	0·825	0·785
0·4	0·668	0·286	0·686	0·685
0·3	0·593	0·292	0·592	0·585
0·2	0·524	0·323	0·523	0·485
0·1	0·377	0·277	0·377	0·385

We see that the values given by $C_1 = \frac{m}{r^2 k}$ and $C = P + C$ are almost identical as the theory demands. But if P is supposed to be constant according to Holmgren, and if the average value is taken for P, we find that the values no longer agree so closely. This difference is better demonstrated by the graphic method in constructing the C-C_1-curve (Fig. 3).

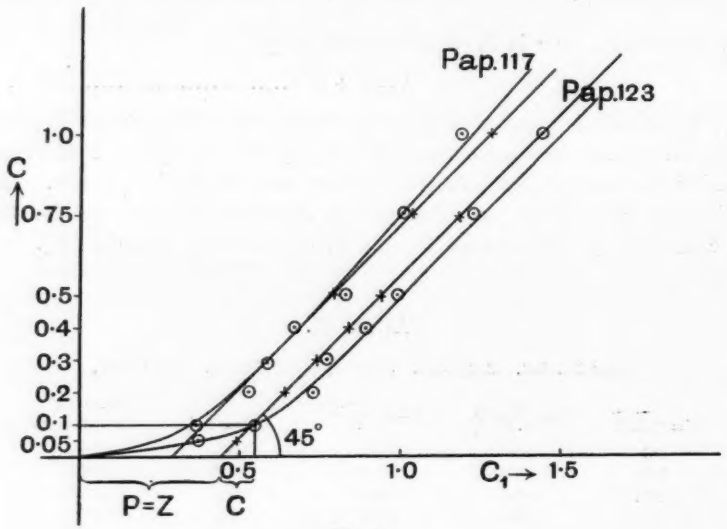

Fig. 3.

It is evident that $C_1 = P + C$ represents a straight line, which cuts the C_1-axis at a distance P from the origin and the inclination of which must be 45°.

$C_1 = \frac{m}{r^2 k}$ represents a parabola, by means of which it is possible to demonstrate the fact that for $C = 0$, C_1 must also be 0, a fact which is not expressible by $C_1 = P + C$, P supposed to be constant.

In reality, as P varies with the concentration, the equation $C_1 = P + C$ represents a system of parallel straight lines and the points indicating the relation between C and C_1 follow the course of a parabola cutting this system.

Nevertheless, the disagreement between Holmgren's supposition and the observed values is so slight, that for concentrations not under 0·1 % his method is for practical purposes very useful on account of its extraordinary facility. To demonstrate the very close agreement of the calculation by Holmgren's formula (2) with the reality I give the following table:

TABLE XI.

Paper 123. $a = 0{\cdot}116$ gr.

C = % HCl	$P = 0{\cdot}441$ (const.) $C = P \frac{r^2}{R^2 - r^2}$	$m = \sqrt[0{\cdot}273]{\frac{r}{18{\cdot}88}}$ $\frac{m}{a}$ = %
2·0	2·87	1·4
1·0	1·02	0·989
0·75	0·69	0·77
0·5	0·43	0·53
0·4	0·36	0·44
0·3	0·28	0·33
0·2	0·16	0·18
0·1	0·097	0·084
0·05	0·068	0·048

With regard to a possible combination of the dye and the acid in the paper, the amount of Congo-red or methyl-orange can be approximately calculated. Bayliss [1906] has shown in regard to Congo-red, that the staining of paper is an adsorption process. The temperature-coefficient of the reaction-velocity is so low, that at room temperature at least 24 hours are required for the attainment of equilibrium. I used a clear looking alcoholic solution of Congo-red (0·04 %) and of methyl-orange (0·1 %) always at room-temperature, and dipped the piece of paper into these solutions only for a few seconds, then allowing the alcohol to drain off and drying the paper at 37°. It can therefore be presumed that the amount of dye adsorbed is very small, and if it be assumed that in so short a time only that amount of Congo-red (or methyl-orange) can be adsorbed which was present in the solution filling the interstices of the paper-fibres, the possible error is certainly very small.

Of an 0·04 % alcoholic Congo-red solution each cmm. contains 4.10^{-7} g. dye,

or in case of 0·1 % methyl-orange $0{\cdot}1 . 10^{-7}$ g. dye. As already shown, the volume of the interstices, which belongs to a surface of πr^2, has been found to be $r^2 . k$. According to Table I r was found = 10 mm. in case of an 0·1 % HCl, which gives $r^2 . k = 20{\cdot}9$ or 21 cmm. Therefore the amount of dye adsorbed in these 21 cmm. is in case of Congo-red $84 . 10^{-7}$, and in case of methyl-orange $2{\cdot}1 . 10^{-7}$. The same consideration gives $2{\cdot}1 . 10^{-7}$ as the amount of an 0·1 % HCl in the same volume of interstices.

These figures are mentioned although I was not able to prove that any chemical action resulting in a perceptible decrease in the amount of free acid took place between dye and acid. No difference in the measurement of the radii of the acid-circles could be observed on varying the concentration of the indicator-solution. Holmgren, who compared the effect of 1·0 and 0·1 % solutions of Congo-red in water on the extent of the surface produced by allowing a drop of acid to fall on paper under similar conditions, thought it also very improbable that the amount of dye by itself plays any important part.

But if the effect of adsorption in unstained paper is compared with that obtained in stained paper, a difference can be observed.

Experimenting under the conditions described in the introduction, one observes in unstained paper that the extent of the water-zone exceeds by 2·5 mm. that found in paper stained by the alcoholic indicator-solutions. This difference in the extent of the water-zone is constant, when the concentration of the acid employed varies, a fact which agrees with the circumstance that R is independent of the concentration.

The extent (r) of the acid-spot is also less in stained paper than in unstained, but according to observation this difference increases the more the acid has been diluted, which favours the idea that a chemical action occurs between acid and indicator.

The weight of about 500 gr., which I used to prevent the paper from wrinkling, influences the extent of the water-zone, but the enlargement caused by the pressure is very insignificant and does not exceed 0·1 mm.

In studying the influence of pure alcohol on the paper regarding the adsorption of hydrochloric acid and the capillary extension of water, experiments showed that pure alcohol has an inhibiting action on water as well as on diluted acid.

I may suggest that the fibres of the paper when treated by alcohol, whether pure or combined with dye, are inhibited or prevented from swelling by water, and thus the interstices become larger, which would explain the observation that the radii become smaller.

To apply these considerations to the method which involves the measurement of the capillary height in strips of paper the different volume of liquid must be taken into account. Skraup and his co-workers [1910] were able to show that the amount of water raised in strips of filter-paper is different in different parts of the strip, decreasing in a hyperbolic manner [p. 887]. Whether a similar decrease of the amount of water occurs in the case of a drop producing a circle on the filter-paper is not proved, and I think it very improbable, believing that this phenomenon depends upon gravitation and therefore upon the inclination of the strip, the influence of which is shown by Goppelsroeder [1909] in regard to the capillary height.

I found also in strips of paper that the fact whether they are untreated, stained by alcoholic Congo-red solutions or only treated by pure alcohol, must be taken in account. Thus under the same experimental conditions distilled water rises quicker in the unstained paper and the contrary takes place in case of a dilute hydrochloric acid.

The following table contains measurements of capillary heights in mm., the time having been constant for each experiment, but I could not find any satisfactory explanation of the phenomenon.

		Paper 123 untreated	Treated by	
			Pure alcohol	Congo-red
Diluted HCl	Water	59	71	71
	Acid	50	59	59
Distilled water		85	105	77
		66	78	62
		76	89	69

Holmgren [1908] found that the relation between the capillary heights of the acid and the water increases according to the concentration, and that this relation is constant for the same concentration; Skraup and his co-workers [1910] found for all acids (with a few exceptions) that the stronger the acid the higher is the degree of adsorption and *vice versa*.

I hope later to be able to show how the mathematical considerations described in this paper can be applied also to Goppelsroeder's and Skraup's experimental data obtained by the measurement of the capillary height. But I am conscious of the fact that these formulae are still far from being able to describe all the possibilities in such a complex phenomenon as the adsorption of acids by paper.

Conclusions.

1. Diluted acids produce a ring system when dropped on blotting-paper, the acid remaining behind the water.

2. The radius r of the coloured circle produced by the acid in the paper is connected with the concentration C by an exponential equation of the form $r = \beta . C^n$.

3. The radius R of the water-zone is independent of the concentration and can be determined by the equation $R = \sqrt{\frac{a}{k}}$, a being the volume of the acid drop in question and k being a constant which depends upon the quality of the [stained] paper.

4. The adsorption causes an increase of concentration. The final concentration is found to be a parabolic function of r.

5. The increment of concentration varies with the initial concentration.

6. Holmgren's calculation, which assumes that the increment of concentration is a constant dependent only upon the quality of the paper, is theoretically incorrect, but it has been shown that it may be useful for practical purposes.

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XXV. A NOTE ON THE HOPKINS AND COLE MODIFICATION OF THE ADAMKIEWICZ TEST FOR PROTEIN.

By VERNON HENRY MOTTRAM.

From the Physiological Laboratory, University of Liverpool.

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Introduction.

The following brief piece of research is the outcome of an unexpected failure in class-work to obtain the well-known reaction with "reduced oxalic" acid [Hopkins and Cole, 1901, 2] protein and concentrated sulphuric acid. In the previous experience of the writer at Cambridge and elsewhere, the test had never failed, and a high estimate of its reliability had been formed, so that this case, when nothing but a yellow to a yellow-brown ring was obtained, needed investigation.

It was soon found that the failure was due to an accidental admixture of the bench sulphuric acid with nitric acid, but during, and arising out of, the search for the cause, the phenomena recorded below were observed.

It became obvious that three disturbing factors were involved: (*a*) the presence of small quantities of impurities in the sulphuric acid, (*b*) the absence of traces of impurities, and (*c*) physical factors influencing the rise and fall of temperature of the reacting fluids. These have been investigated in turn.

(1) *The presence of impurities in supraminimal amounts.*

The cause of the trouble recorded above was the presence in the sulphuric acid of nitric acid.

The test, as has been pointed out by Salkowski [1888] and Hopkins and Cole [1901, 1], and confirmed by Rosenheim [1906], is spoilt by oxidising agents. This occurs at small concentrations, especially if, as a criterion, rather than the formation of a ring, the colour of the resultant mixture be

taken[1]. Thus a final concentration of 1 part by volume of hydrogen peroxide (10 vols. comm.) in 50, of 1 part by weight of ferric chloride in 1000, of 1 part by weight of potassium chlorate in 25,000, of 1 part by volume of concentrated nitric acid in 25,000 or of 1 part by weight of sodium nitrite in 50,000 parts by volume prevents the formation of a violet or purple coloration.

In all cases except one (nitric acid) a yellow ring is formed, which, on shaking the tube gently from side to side, spreads throughout the mixture. With nitric acid there appears a purple ring above the yellow ring at the junction of the fluids. When the test tube is shaken the purple ring moves up the test tube, is overtaken by the yellow coloration and is destroyed. In no case is there charring.

For laboratory practice, therefore, if, instead of a lilac, mauve or violet coloration, a yellow, a red, a red-purple or a brown ring is formed, it is probably due to contamination of the sulphuric acid with oxidising agents. Commercial sulphuric acid, to judge by the samples investigated, never has enough oxidising agents present to prevent the reaction.

(2) *The influence of oxidising agents in minimal amounts*

In the course of a large number of experiments it has become clear that Rosenheim's statement that an oxidising agent is necessary to the reaction is justified. The observations on which this is based are as follows (for experimental details consult Appendix, § 2):

(*a*) Some very pure samples of sulphuric acid (supplied by Messrs Evans, Lescher and Webb, Messrs Baird and Tatlock, Liverpool, and Messrs Towers and Sons, Widnes and Liverpool) give with freshly made or with old "reduced oxalic," whether strong or weak, or with Benedict's magnesium-reduced oxalic [1909] a lilac or lilac-mauve colour instead of the typical violet (see Appendix, § 2, Exp. LVIII etc.). But if traces of oxidising agents are added to the "reduced oxalic" the blue-violet or violet develops at once (Appendix, § 2). The optimal concentration is, for hydrogen peroxide (10 vols. comm.) *circa* 1 part by volume in 1000 of the resulting mixture, for ferric chloride 1 part by weight in 20,000 and for sodium nitrite 1 part in 375,000.

(*b*) Subsequent addition of oxidising agents after the lilac or lilac-mauve coloration with pure sulphuric has developed converts this colour to a blue-violet of great density and beauty (Appendix, § 1, Exps. LII—LIV)[2]. This

[1] For experimental details see Appendix, § 1, Exps. I, II, XXX, LII—LV.

[2] The paradox of the greater stability of the colour when once formed towards oxidising reagents is simply explained as the decrease in velocity of a chemical reaction concurrent with fall of temperature.

does not always take place if the reacting fluids have cooled. But in this case, warming, with previous or subsequent addition of an oxidising agent, yields the typical blue-violet (Appendix, § 3 and § 6). Continued heating alters the test through a series of colours typically the result of the addition of increasingly greater quantities of oxidising agents to the "reduced oxalic" (Appendix. Compare § 3, with § 2 Exp. LIX and § 4 Exp. LXIV). Obviously change in colour is a sign of advancing oxidation.

(*c*) Commercial sulphuric acid will withstand the addition of less oxidising agent than will pure sulphuric acid if the test is to be typical. The reaction is not improved but spoilt by the addition of even small traces (Appendix, § 4, Exp. XXXVI, etc.).

(*d*) Commercial sulphuric acid will withstand the addition of more formaldehyde than will pure sulphuric acid (Appendix, § 4, Exp. XLI and XLII).

(*e*) If the violet fluid resultant from the use of commercial sulphuric acid be boiled, the colour changes seen in the parallel example quoted in section (*b*) above are imitated with exactitude (Appendix, § 4, Exp. LXIV). Oxidation by means of the oxidising agents present in the commercial sulphuric acid is proceeding.

These five observations accord well with Rosenheim's theory that oxidising agents are necessary to the reaction. They suggest that a coloured substance arises from the oxidation of a tryptophane-formaldehyde product formed from the protein and that further oxidation of this blue-violet substance modifies this colour and ultimately destroys it. We may assume that this reaction proceeds at a velocity very much greater at high temperatures than at lower temperatures and that, in the optimal conditions of the test, the cooling is rapid enough to prevent the oxidation going too far.

That there is a change proceeding at a definite rate may be seen by carrying out the test under conditions in which a high temperature is not attained, or if attained, is rapidly reduced. For instance the initial colour may be profoundly modified in a very wide tube, or by cooling the test tube under the tap. A rose colour at first develops, but in the first case after the test tube has stood some time the colour has deepened to mauve and after a day to blue-violet. It is doubtful if this be due to the oxygen of the air, for no observable difference was found when the test was carried out under an atmosphere of carbon dioxide.

To the question whether a balance of formaldehyde and oxidising agents is necessary [see Rosenheim, 1906] (another case of the two classical antagonistic principles) my experiments give no certain answer. If these be

both in the "reduced oxalic" and in optimal equipoise, then reduction of the strength of the "reduced oxalic" should render the equipoise more susceptible to tilting agencies. Oxidisers should have more effect in the presence of weak "reduced oxalic" than of strong. There is no evidence to offer that this is the case (App. § 5, Summary of Exps. XVIII to XXXV).

(3) *The influence of physical conditions.*

It was thought that physical conditions, such as the size and shape of the test tube, area of reacting fluids compared with their volume, and rapidity with which the fluids reached their maximal temperature and subsequently cooled would affect the results. This is indeed so but, except in the extreme cases given above, is not of much importance. Thick test tubes give a lighter result than thin; very narrow than normal size; and very large tubes with inadequate amounts of fluid than normal tubes or large tubes with adequate amounts. Cooling under the tap produces a rose colour shot with green which subsequently, unless oxidising agents are added, does not darken much. In three days it is lilac in colour (see App. § 6).

One reason for the different colours observed when different volumes of the reagents are used is to be found in the dichromatism of the coloured substance. It is lilac in thin layers or great dilutions, and bluer in thicker layers and greater concentrations. This, of course, has its parallel in its absorption band in the spectrum. A mixture that was lilac showed an absorption band from λ 592 to λ 547, on adding a drop of ferric chloride the band stretched from λ 646 to λ 511, diluting the mixture with pure sulphuric acid brought the band to its original dimensions and the mixture to its original colour. On warming the diluted mixture, with consequent alteration in colour to purple, the band stretched from λ 610 to λ 531, and the blue and violet were reduced in intensity. This band is identical with one described by Hopkins and Cole [1901, 1].

Finally, the different colours observed with weaker solutions of protein are due to the oxidation of the chromogen beyond the optimum. Weaker solutions give with either pure or commercial sulphuric acid results that are redder in tint than the controls. This is not dichromatism, for dilution with sulphuric acid leaves the control many degrees bluer than the others. The relation of this phenomenon to the others given above is clear if we assume progressive oxidation.

Summary and Conclusions.

(1) The modification of the Adamkiewicz reaction introduced by Hopkins and Cole fails in the presence of small amounts of oxidising agents.

(2) But traces of these improve the reaction when carried out with "pure" sulphuric acid.

(3) Probably, therefore, the result with "commercial" sulphuric acid is due to the presence of contained oxidising agents.

(4) Whether the trace of oxidising agent which gives the lilac to mauve tint with "pure" sulphuric acid is in the reduced oxalic or in the sulphuric acid cannot be settled. In view of Miss Homer's work [1911] it is probably in the sulphuric acid.

(5) The resultant coloration of the mixture depends on the extent of a combination of a chromogen with oxygen. This combination has a definite rate of reaction which is naturally more rapid at high temperature and at greater concentrations of the oxidising agent.

(6) It is therefore best to keep the volumes and percentage compositions of the reacting fluids as constant in proportion as possible and their absolute measure should depend on the size of the test-tube[1].

(7) The following method of carrying out the test yields excellent results: 1 c.c. of strong "reduced oxalic" acid (Benedict's "reduced oxalic" will do just as well) is mixed with 1 c.c. of 1 % Witte's peptone, and then 2 c.c. of sulphuric acid are run down the side of the test-tube which is held as near horizontal as convenient. The fluids are then rapidly mixed by shaking from side to side with the test-tube vertical. The quantities given are for a test-tube 1·5 cm. in diameter (the usual laboratory size). Subsequent addition of one drop of 1 % $FeCl_3$ solution usually increases the density and the blueness of the coloration.

The quantities given may be varied greatly and yet a good coloration be obtained. 1 c.c. or 5 c.c. sulphuric acid to 2 c.c. of the mixture yield an unmistakable result and the quantities may be guessed and not measured. More sulphuric acid gives a bluer result than less.

[1] I can see no reason for abandoning the excellent reagent known as "reduced oxalic" acid in favour of formaldehyde and doctored sulphuric acid. As long as the test is performed under conditions that lead to a maximal development of heat, all samples of sulphuric acid give a reasonable coloration. It is only when the reacting fluids are cooled or contaminated that an imperfect result is obtained; whereas the balance of oxidising agent and formaldehyde is easily tilted in an adverse direction, especially in the hands of students.

"Reduced oxalic" acid is a reagent easily made, particularly now that magnesium powder is a requisite in the biochemical laboratory. It does not diminish in strength even when more than a year old.

The details of the experiments on which the statements above made are based are relegated to an appendix.

I have to thank Messrs Hopkins and Cole for reading through the MS. of this paper and for suggestions they have made. Their explanation of the phenomena recorded must be left to a future communication from them.

Appendix.

1. *Experiments on the destructive effect of oxidising agents.*

In most of the experiments performed the production of a coloration was taken as a criterion and not the formation of a ring. In the experiments based on the plan of Exp. XXX (*v. inf.*)—the majority—all the test tubes were prepared before the shaking of the contents took place. This naturally does not give a maximal rise of temperature. The first test tube has to wait a minute or so before the fluids it contains are mixed and the heat evolved by the natural admixture at the junction of the fluids is to some extent lost. This exaggerates the influence of oxidising agents as adjuvants of the reaction, and decreases the influence as deterrents.

Exp. I. Equal parts stock solution of "reduced oxalic" undiluted and (*circa*) 1 % acid albumin mixed.

Mixture	Pure sulphuric	Oxidiser	Remarks
25 c.c.	25 c.c.	1 drop conc. HNO_3	Yellow colour, faint effervescence.
25 c.c.	25 c.c.	Small amt. $FeCl_3$	Normal violet, disappeared on adding excess $FeCl_3$.
25 c.c.	25 c.c.	1 c.c. 10 vols. H_2O_2	Light yellow coloration.

Exp. II. Same mixture as above.

Mixture.	Comm. sulph.	Oxidiser	Remarks
2 c.c.	2 c.c.	Nil	Violet ring.
2 c.c.	2 c.c.	2 drops 0·01 % HNO_3	Yellow ring.
2 c.c.	2 c.c.	1 drop ,, ,,	Faint violet above, yellow ring below.
2 c.c.	2 c.c.	(1 drop water)	Violet ring.

Exp. XXX. Mixture 1 % peptone and strong "reduced oxalic." Equal parts.

Mixture	H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks	Coloration
2 c.c.	0 drops	5 drops	2 c.c.	Ring not clear.	Lilac-mauve.
2 c.c.	1 ,,	4 ,,	2 c.c.	Good ring.	Clear violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Good ring.	Purple, then rose.
2 c.c.	3 ,,	2 ,,	2 c.c.	Vanishing ring.	Light red.
2 c.c.	4 ,,	1 ,,	2 c.c.	Vanishing ring.	Yellow.
2 c.c.	5 ,,	0 ,,	2 c.c.	Vanishing ring.	Straw colour.

Throughout the experiments based on this plan the same pipettes were kept for each reagent. The drops were frequently estimated and their

average volume was 0·04 c.c. Of course in the measurement of the above volumes, whether large or small, no great accuracy was obtained. A 1 c.c. pipette has a high percentage error (*circa* 5 %).

Exp. LII. $NaNO_2$. *Same mixture as in Exp. XXX.* In the experiments following, not the formation of a ring, but the final coloration is used as a criterion.

Mixture	0·1 % $NaNO_2$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac coloration (deep blue-violet on adding drop of $NaNO_2$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Dense purple coloration.
2 c.c.	2 ,,	3 ,,	2 c.c.	Red coloration.
2 c.c.	3 ,,	2 ,,	2 c.c.	Orange coloration.
2 c.c.	4 ,,	1 ,,	2 c.c.	Yellow coloration.
2 c.c.	5 ,,	0 ,,	2 c.c.	Yellow coloration.

Exp. LIII. $FeCl_3$.

Mixture	4 % $FeCl_3$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (dense blue on adding 1 drop $FeCl_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple coloration.
2 c.c.	2 ,,	3 ,,	2 c.c.	Claret ,,
2 c.c.	3 ,,	2 ,,	2 c.c.	Red-brown ,,
2 c.c.	4 ,,	1 ,,	2 c.c.	Brown ,,
2 c.c.	5 ,,	0 ,,	2 c.c.	,, ,,

Exp. LIV. $KClO_3$.

Mixture	0·2 % $KClO_3$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac coloration (purple on addition of 1 drop $KClO_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple coloration.
2 c.c.	2 ,,	3 ,,	2 c.c.	Yellow ,,
2 c.c.	3 ,,	2 ,,	2 c.c.	,, ,,

Exp. LV. HNO_3.

Mixture	HNO_3 2 vols. per 1000	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (no deepening on adding HNO_3).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple.
2 c.c.	2 ,,	3 ,,	2 c.c.	Red-brown.
2 c.c.	3 ,,	2 ,,	2 c.c.	Yellow.
2 c.c.	4 ,,	1 ,,	2 c.c.	Yellow.
2 c.c.	5 ,,	0 ,,	2 c.c.	Yellow.

In this experiment a purple ring was formed above the junction of fluids with a yellow ring beneath. On gently shaking, the above colours were produced.

A number of other experiments on similar lines were performed with similar results.

2. *Experiments on the beneficial effect of traces of oxidising agents.*
(See also Experiments immediately preceding.)

Exp. VIII. Peptone mixture.

	Mixture	10 % H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks
(*a*)	2 c.c.	0·0 c.c.	1·0 c.c.	2 c.c.	Ring obtained in all. On shaking, (*a*) (i) and (ii) were the most violet, (*b*) the deepest and (*c*) the weakest. After two hours (*b*) was nearly bleached and (*c*) yellow.
	2 c.c.	0·0 c.c.	1·0 c.c.	2 c.c.	
(*b*)	2 c.c.	0·5 c.c.	0·5 c.c.	2 c.c.	
	2 c.c.	0·5 c.c.	0·5 c.c.	2 c.c.	
(*c*)	2 c.c.	1·0 c.c.	0·0 c.c.	2 c.c.	
	2 c.c.	1·0 c.c.	0·0 c.c.	2 c.c.	

Exp. IX.

2 c.c.	0 drops	5 drops	2 c.c.	Blue-violet coloration.
2 c.c.	1 ,,	4 ,,	2 c.c.	Dense violet, showed up most rapidly.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple.
2 c.c.	3 ,,	2 ,,	2 c.c.	Purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Red-purple.
2 c.c.	5 ,,	0 ,,	2 c.c.	Red-purple.

A number of other experiments with different concentrations of H_2O_2 gave similar results. Exp. XXI is quoted. Strong reduced oxalic diluted five times and then mixed with an equal volume of 1 % Witte's peptone.

Exp. XXI.

Mixture	4 % H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Pale mauve.
2 c.c.	1 ,,	4 ,,	2 c.c.	Violet-purple. Three times as dense.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple.
2 c.c.	3 ,,	2 ,,	2 c.c.	Rose-purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Purple-rose.
2 c.c.	5 ,,	0 ,,	2 c.c.	,, ,,

Exp. LVIII. Mixture: 1 part freshly made reduced saturated oxalic and 1 part 1 % Witte's peptone.

Mixture	0·5 % $FeCl_3$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac. (Deepened to royal blue on subsequent addition of $FeCl_3$).
2 c.c.	1 ,,	4 ,,	2 c.c.	Deep violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Clear violet.
2 c.c.	3 ,,	2 ,,	2 c.c.	Purple-violet.
2 c.c.	4 ,,	1 ,,	2 c.c.	Purple.
2 c.c.	5 ,,	0 ,,	2 c.c.	Red-purple.

Exp. LIX. Mixture as above.

Mixture	0·04 % $NaNO_2$	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (deepening remarkably on addition of $NaNO_2$ solution).
2 c.c.	1 ,,	4 ,,	2 c.c.	Violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple-violet (strongest at first, bleaching later).
2 c.c.	3 ,,	2 ,,	2 c.c.	Purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Brown-purple.
2 c.c.	5 ,,	0 ,,	2 c.c.	Purple-brown.

Very many other experiments were performed, some of which are quoted in other sections, with the same results.

3. *Experiments on the subsequent addition of oxidising agents.*

As will be seen in a number of the above experiments, addition of oxidising agents after the lilac coloration was formed produced a deep blue coloration. In some earlier experiments on cooling the fluids while the mixture was being made, it was noticed that the cold mixture of sulphuric acid reduced oxalic and peptone did not turn blue on the addition of ferric chloride, but after the addition warming gave the intense blue coloration. This on standing turned violet. Other tests showed that rewarming the mixture and then adding ferric chloride produced the same result, and that further warming, or rather boiling, made the colour proceed through violet, purple, to red and ultimately bleached it almost completely. Further notes will be found on similar results obtained in the experiments on the effect of cooling the test tube while the sulphuric and the other reagents are mixing (App. § 6).

4. *Comparison between pure and commercial sulphuric acid.*

Exps. XLI and XLII.

Mixture	0·04 % formaldehyde	H_2O	2 c.c. Commercial sulphuric	2 c.c. Pure sulphuric
2 c.c.	0 drops	5 drops	Violet	Lilac-mauve (gives blue ring on running in H_2O_2).
2 c.c.	1 ,,	4 ,,	Dense violet	Olive green.
2 c.c.	2 ,,	3 ,,	Brown-violet	,, ,,
2 c.c.	3 ,,	2 ,,	Brown-purple	,, ,,
2 c.c.	4 ,,	1 ,,	Purple-brown	,, ,,
2 c.c.	5 ,,	0 ,,	Light purple-brown	,, ,,

Exp. LXIV. Experiments on same plan as above made with different samples of acid.

B. & T. commercial sulphuric	Purple-violet	On heating passed through purple to purple-red and brown.
T. ,, ,,	Purple-violet	
E. L. W. ,, ,,	Purple-violet	
T. pure ,,	Lilac	On heating slowly altered to purple-brown obtained in XLI, 6.
E. L. W. ,, ,,	Bluish-lilac	

Exp. LXI. When various samples of acids, commercial and pure, were used, but to 2 c.c. of mixture and 4 drops of H_2O in each case 1 drop of 0·04 % $NaNO_2$ was added, the following results were obtained:

Sulphuric		Remarks
B. & T. pure,	2 c.c.	Blue-violet.
B. & T. comm.,	,,	Purple.
T. pure,	,,	Blue-violet.
T. comm.,	,,	Purple.
E. L. W. pure,	,,	Blue-violet.
E. L. W. comm.	,,	Purple.

Exps. XXXVI to XL and LX with varying strengths oxidising agents added showed that the commercial sulphuric used had the optimal amount of oxidising agents present. Compare with tests in §§ 2 and 3. As an example XXXVIII is quoted.

Mixture	2 % 10 vols. H_2O_2	H_2O	Commercial sulphate	Coloration
2 c.c.	0 drops	5 drops	2 c.c.	Violet (densest).
2 c.c.	1 ,,	4 ,,	2 c.c.	Purple-violet.
2 c.c.	2 ,,	3 ,,	2 c.c.	Purple.
2 c.c.	3 ,,	2 ,,	2 c.c.	Red-purple.
2 c.c.	4 ,,	1 ,,	2 c.c.	Purple-red.
2 c.c.	5 ,,	0 ,,	2 c.c.	Red-brown.

5. *Experiments on the supposed antagonism between formaldehyde and oxidising agents and on the occurrence of the latter in reduced oxalic acid.*

"weak" Hopkins' reagent = reduced saturated oxalic diluted 5 times.
"normal" ,, ,, = ,, ,, ,, ,, $2\frac{1}{2}$,,
"strong" ,, ,, = ,, ,, ,, undiluted.

The experiments were carried out as usual, six tests in each, with increasing strengths and amounts of hydrogen peroxide; thus:

Exp. XXXIII. Mixture of equal parts of strong Hopkins' reagent and 1 % peptone.

	Mixture	2 % 10 vols. H_2O_2	H_2O	H_2SO_4	Coloration
(*a*)	2 c.c.	0 drops	5 drops	2 c.c.	Lilac-mauve.
(*b*)	2 c.c.	1 ,,	4 ,,	2 c.c.	Violet-mauve.
(*c*)	2 c.c.	2 ,,	3 ,,	2 c.c.	Violet } densest.
(*d*)	2 c.c.	3 ,,	2 ,,	2 c.c.	Violet }
(*e*)	2 c.c.	4 ,,	1 ,,	2 c.c.	Purple-violet.
(*f*)	2 c.c.	5 ,,	0 ,,	2 c.c.	Purple.

Summarising the results it was found:

	Densest colour		
10 vols. H_2O_2	Strong reagent	Normal reagent	Weak reagent
10 %	(*b*) (violet)	(*b*)	(*b*)
4	(*b*) & (*c*) (purple-violet)	(*b*)	(*b*)
2 %	(*c*) & (*d*) (violet)	(*d*) & (*e*)	(*c*)
1·3 %	(*d*) (violet)	(*d*)	(*e*)
1 %	(*b*) to (*d*) (much the same colours)	—	(*f*)

No evidence is seen of oxidising agents deflecting the optimum towards nil concentration when weak Hopkins' reagent is used. This should be the case if oxidising agents are in the reduced oxalic.

6. *Experiments on effect of preventing, or of accelerating, rise to maximal temperature.*

Exp. LXV.

Mixture	Pure sulphuric	Procedure	Coloration
2 c.c.	2 c.c.	Shaken under cold tap	Pale rose with green fluorescence.
2 c.c.	2 c.c.	,, ,, warm tap (45° C. *circ.*)	Rose.
2 c.c.	2 c.c.	Shaken in air	Lilac-mauve.
2 c.c.	2 c.c.	,, ,, Bunsen flame	Violet-mauve, changing to brown-violet.

Repeated first experiment left to stand...		...	Mauve after three days.
,,	,,	,, added 1 drop 4 % $FeCl_2$	Pale rose slowly alters to deep rose, lilac-mauve and on gentle warming to violet.
,,	,,	,, very thoroughly cooled, added 1 drop 4 % $FeCl_3$	Slowly darkening in about an hour to lilac-mauve.

7. *Experiment on dilution of peptone.*

	Peptone	Strong Hopkins' reagent	Sulphuric	Coloration.
(*a*)	1 c.c. (1 %)	1 c.c.	2 c.c. (pure)	Lilac-mauve.
(*b*)	1 c.c. ,,	1 c.c.	2 c.c. (comm.)	Violet-purple.
(*c*)	1 c.c. (0·2 %)	1 c.c.	2 c.c. (pure)	Pale lilac-violet.
(*d*)	1 c.c. ,,	1 c.c.	2 c.c. (comm.)	Pale purple.
(*e*)	1 c.c. (0·1 %)	1 c.c.	2 c.c. (pure)	Very pale lilac-purple.
(*f*)	1 c.c. ,,	1 c.c.	2 c.c. (comm.)	Light rose-purple.

When (*a*) and (*b*) are diluted with pure sulphuric the result is bluer than any parallel experiment with weaker peptone. Addition of commercial sulphuric acid to (*b*) produced a redder colour than addition of pure sulphuric acid to (*b*). These observations accord with the theory of progressive oxidation of a chromogenic substance by oxidising agents in the sulphuric acid.

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XXVI. THE USE OF LITMUS PAPER AS A QUANTITATIVE INDICATOR OF REACTION.

By GEORGE STANLEY WALPOLE.

From the Wellcome Physiological Research Laboratories, Herne Hill, S.E.

(*Received April 7th, 1913.*)

In dealing with nearly neutral solutions of feebly dissociated electrolytes, especially when they are highly coloured, it is frequently convenient to gauge reaction by the use of litmus paper, and so avoid either electric measurement or the introduction of a fluid indicator into a comparatively large volume of fluid. And it has been found that if suitable precautions are taken quite reliable information can be obtained as to the $H^{\cdot}$ ion concentration of such fluids in this way. The apparently contradictory results obtained in such cases by using different commercial samples of litmus paper, immersing the paper for a short or a long time in the fluid, using one drop on a piece of paper or a small piece of paper in a considerable volume of fluid, and so on, are capable of simple interpretation.

The precautions to be observed are all those which apply to the use of litmus solution with the addition of those necessitated by the fact that it is applied to paper. As an example of the former class the "neutral salt effect" may be mentioned: the "time factor" is typical of the latter.

Experimental.

The following solutions were used:

Ammonia-ammonium chloride mixtures. A solution of ammonium chloride and ammonia was prepared so as to be approximately 3 N with respect to the former constituent and 3/32 N with respect to the latter. Dilutions with boiled distilled water were made ×2, ×4, ×16, ×32. According to Fels [1904] these solutions should have the same P_H^+ value at 18° C. The values obtained on examination in the hydrogen cell using a calomel electrode were 8·14, 8·14, 8·13, 8·14 respectively, while 8·12 was that obtained for the undiluted material.

Acetic acid-sodium acetate mixtures. A solution of commercial sodium acetate 0·61 N, containing acetic acid corresponding to 0·61/64 N was diluted ×1, ×2, ×8 and ×16. These solutions should have the same P_H^+ value very closely. The values found were 6·25, 6·20, 6·27, 6·23.

Neutral phosphate solution. This was a mixture of 68·8 c.c. of Sörensen's N/15 Na_2HPO_4 solution, and 131·2 c.c. of his N/15 KH_2PO_4 solution. The P_H^+ value determined was 7·05. The figure at present accepted for absolute neutrality is $P_H^+ = 7·07$ [Sörensen, 1909].

Solutions of serum globulin and Witte peptone were also examined, but they presented, in addition to the phenomena observed with solutions free from proteins and their primary disintegration products, others of a more complex and different order. For this reason it was decided to deal with them more fully in a separate communication and cite here only one or two typical cases.

Litmus solution. The reaction between litmus solution and the solution with which it is mixed is practically instantaneous. In saline solutions it gives an indication of the reaction of the solution subject only to a correction for the "neutral salt effect": for it is well known that, for solutions of the same $H^{\cdot}$ ion concentration, indicators in the presence of larger quantities of neutral salt give slightly different colours from those which they give when little salt is present [Sörensen, 1912].

The litmus tincture used was itself apparently neutral, or very nearly so, since it gave almost exactly the same tint when added in the same quantity to equal volumes of boiled distilled water and neutral phosphate solution.

The ammonia-ammonium chloride mixtures all gave precisely the same tint as far as could be observed with litmus solution, with the exception of the ×32 dilution, 100 c.c. of which required 0·02 c.c. N/10 NaOH to bring the colour to the same tint as that of the others. The acetic acid-sodium acetate mixtures, on the other hand, gave progressively pinker solutions with litmus tincture as dilution increased. From this observation I think it is legitimate to draw the conclusion that the neutral salt effect is observable in all the acetic acid-acetate mixtures, and only in one case in the ammonia-ammonium chloride mixtures. This is probably due to the fact that the P_H^+ value of the latter mixture is near the alkaline limit of the sensitive range of litmus.

"*Equilibrium tint.*" If a small piece of litmus paper be introduced into a comparatively large volume of any of these solutions it will, after a lapse of a longer or shorter time, assume a definite tint which undergoes no further change. This it is proposed to call the "equilibrium tint" of the paper used in the solution under consideration.

"*Reaction inertia.*" A solution whose H˙ ion concentration is but little affected by the addition of H˙ or OH′ ions, say in the form of hydrochloric acid or caustic soda, can be said to have a greater "reaction inertia" than pure water or a dilute solution of a completely dissociated electrolyte, whose H˙ ion concentration would be profoundly affected by such an addition.

The above acetic acid-sodium acetate mixtures, although of the same absolute reaction ($P_H^+ = 6{\cdot}2$), differ very much in reaction inertia, as is in fact patent from their composition. The number of c.c. of N/10 NaOH solution required to bring 100 c.c. of any one solution from $P_H^+ = 6{\cdot}2$ to $P_H^+ = 8{\cdot}1$ may be for the purpose of this note considered as a numerical expression of this property. A 50 c.c. sample of each dilution being taken and equal quantities of litmus tincture added to each they presented different colours but had $P_H^+ = 6{\cdot}2$ in each case as described above. A 50 c.c. sample of an ammonia-ammonium chloride mixture to which an equal quantity of litmus tincture was added furnished a standard tint $P_H^+ = 8{\cdot}1$. The acetic acid-acetate samples were then titrated with N/10 NaOH to this standard. The results are tabulated:

Acetic acid-sodium acetate mixture.

Dilution	Reaction inertia
1	8
2	4
8	1
16	0·5

In a similar manner titrations of the ammonia-ammonium chloride mixtures were undertaken from $P_H^+ = 8{\cdot}1$ to $P_H^+ = 6{\cdot}2$ using N/10 HCl, and from these results a similar table may be prepared. The standard pink tint ($P_H^+ = 6{\cdot}2$) was that of the undiluted acetic acid-acetate mixture.

Ammonia-ammonium chloride mixture.

Dilution	Reaction inertia
1	90
2	45
4	22
16	6
32	3

With N/15 phosphate mixtures it was found that 20 c.c. of a solution $P_H^+ = 8{\cdot}1$ required 0·9 c.c. N HCl to bring the reaction to $P_H^+ = 6{\cdot}2$. The "reaction inertia" for N/15 phosphate solution may then be taken as $0{\cdot}9 \times 5 \times 10 = 45$.

Observations with Litmus paper.

Four commercial varieties of litmus paper were examined, each providing a "red" and a "blue" paper. Three brands were glazed with the litmus applied to one side only, and one soft and absorbent.

Of the three that were glazed, No. 1 was dyed heavily and apparently loaded with a material of considerable "reaction inertia"; No. 2 was not so heavily dyed though the grain of the paper was similar; No. 3 was faintly dyed on a perfectly smooth paper, presenting a homogeneous appearance. The unglazed paper was labelled No. 4.

Colour tint of paper after 24 hours immersion. Small pieces of each paper about 25 mm.^{2} area were immersed for 24 hours in 240 c.c. of ammonia-ammonium chloride solution of each dilution. At the end of that time all the pieces of paper were of the same tint.

Only one difference could be observed. The heavily loaded paper, No. 1 (blue) was more deeply dyed in the more concentrated solutions than in those more dilute. The adsorption equilibrium seems to shift in the direction of greater adsorption of dye with increasing concentration of salt at constant reaction. A special experiment ratified this conclusion. Pieces of paper No. 1 blue after prolonged immersion in solutions $P_H^+ = 8{\cdot}1$ containing 15, 7·5, 3·8, 1·9, 0·5 per cent. NaCl gave up progressively more dye to the solution as dilution increased, though in similar solutions of $P_H^+ = 6{\cdot}1$ no such change could be definitely observed.

Paper No. 1 red immersed in N/15 phosphate solutions $P_H^+ = 4{\cdot}5$, 7·1, 9·2 respectively gave up very markedly increased quantities of litmus to the solution with increasing alkalinity. In parallel experiments the litmus found in the alkaline solution was 5–6 times that found in the acid solution.

This, in conjunction with many similar observations in this connection, has led me to the conclusion that in similar solutions the adsorption equilibrium changes in the direction of diminished adsorption of litmus by paper in solutions of increasing alkalinity.

In the acetic acid-sodium acetate solutions similarly examined small pieces of each paper gave the same tint after 24 hours immersion, only in one and the same dilution. Passing from one dilution to another it was found that with increasing dilution just that change could be noticed in the equilibrium tint which, as far as could be judged corresponded to the variation in tint with litmus tincture due to the "neutral salt effect" described above.

Observation of rate of change of colour. In the above experiments the

time taken for a piece of litmus paper to acquire its equilibrium tint was seen to vary very considerably. The same paper changed more rapidly in a solution of greater reaction inertia than in one of less reaction inertia in all cases, and, as one would expect, a paper took longer to reach its equilibrium tint when the colour change from its original to its final tint was great. For instance a red paper (No. 1 red) took several hours to come to its equilibrium tint in the most dilute alkaline solution, while a blue paper did so in a few minutes as it was already almost at that tint.

Observation of effect of exposure to the air. It will have been noticed that the two main test mixtures chosen each have a volatile constituent. Pieces of paper that have been immersed for sufficient length of time to assume the equilibrium tint, change that tint rapidly on exposure to the air in such a case. The reddening of blue paper due to the volatilisation of ammonia is naturally more rapid than the blueing of red paper due to acetic acid evaporation.

"*Amphoteric solutions.*" Dealing with approximately neutral solutions a distinction is frequently made between a solution that is "merely neutral" and one that is "amphoteric." Milk, urine, and solutions of phosphates are regarded as typical examples of the latter class and they are said to "turn blue litmus paper red, and red litmus paper blue." What is actually seen may be better described as the "reddening of blue litmus paper and the blueing of red litmus paper." The "merely neutral" solution is one of low reaction inertia. A drop placed on paper wets it but only to a very small extent alters its reaction. The colour of the paper therefore remains unchanged. The "amphoteric" solution, on the other hand, has a high reaction inertia. It usually contains a salt of a feebly dissociated acid or base and, being neutral or nearly so, alters the reaction of litmus paper with which it is brought into contact in the direction of neutrality.

It would naturally be expected that small pieces of paper, red and blue, would, when immersed in a large volume of an amphoteric solution, ultimately assume the same tint. During the first few hours of observation it is seen that the blue paper is becoming progressively redder, and the red paper bluer, but before equilibrium can be attained certain disturbing influences may be encountered. For instance if protein be present the colour of the paper will be interfered with. And, again, since the range of reaction through which the litmus changes is different in the two cases, the losses of litmus to the solution will not be the same. A blue and a red sample of the quickly reacting paper No. 4 were seen to assume very nearly the same tint in a large volume of milk in 24 hours.

Preliminary observations in protein solutions. In solutions of protein, peptone and the like the use of litmus paper has, as far as my experience goes, always indicated the solution to be more alkaline than it really is. For instance an acid 4 per cent. solution of Witte peptone containing 1 per cent. of sodium chloride, and 1 c.c. per cent. of normal hydrochloric acid was found by electric measurement to have $P_H^+ = 6{\cdot}5$. In a tintometer arranged to compensate for the colour of the Witte peptone [Walpole, 1910] and using neutral red as indicator, the P_H^+ value was made out to be the same as that of a phosphate mixture $P = 6{\cdot}8$[1]. A neutral N/15 phosphate solution was prepared ($P_H^+ = 7{\cdot}07$) and a comparison instituted between this solution and the acid Witte peptone solution above. It will have been noticed that neither contains a volatile constituent.

Three papers were taken (No. 4 red, No. 2 blue and No. 3 red) and one slip of each paper dropped into a large volume of each fluid. They were removed at intervals, laid on white paper and examined in diffused daylight.

The unglazed, quickly-reacting paper (No. 4 red) gave with the acid peptone solution a bluer tinge almost at once than the corresponding paper in the phosphate solution and this relation continued indefinitely. The blue paper No. 2 was always bluer in the acid peptone solution: the red paper No. 3 lost most of its colour in the phosphate solution before the progressively bluer paper in the acid Witte peptone solution could match it.

In another case a faintly alkaline 4 per cent. solution of Witte peptone of reaction $P_H^+ = 7{\cdot}3$ gave with papers No. 2 (red and blue), No. 3 (red and blue), No. 4 (red and blue) the same equilibrium tint, as nearly as could be judged, as any of the ammonia-ammonium chloride dilutions examined, which had $P_H^+ = 8{\cdot}1$ in each case.

Examples.

By neglecting to consider these secondary influences it is possible to obtain some bewildering results. For instance we may take two alkaline solutions having the same reaction, $P_H^+ = 8{\cdot}1$; A is a solution of ammonium chloride and ammonia, 3N with respect to the former and 3/32 N with respect to the latter; B is this solution diluted 32 times and the "reaction inertia" of A is about 30 times that of B; C is a neutral phosphate solution;

[1] In the same apparatus using litmus tincture as indicator no real match could be obtained but the indications were that the peptone solution was more alkaline than a phosphate mixture $P_H^+ = 9$.

and *D* is pure water. Red litmus paper No. 1 dipped into the solutions, removed, and examined shows at once a bright blue colour with *A*, red with *B*, a bluish tinge with *C* and red with *D*. Blue paper No. 4 shows blue rapidly turning red with *A*, blue slowly turning not quite so red with *B*, red with *C*, and blue with *D*; while blue paper No. 2 shows with *A* and *B* blue very slowly turning red, and with *C* and *D* slight change towards red, less marked in the latter case.

The following interpretations suggest themselves. With red paper No. 1 the concentrated solution *A* neutralises the acid red material of the heavily loaded paper, and in spite of the glazing shows a pronounced blue colour at once; with *B* the reaction of the absorbed fluid is determined more by the paper than by the substances originally dissolved in the water, and the evaporation of the free ammonia has been almost complete long before any great effect would have been observed even if the paper had been bathed in an excess of solution. Solution *C* gives the neutral tint appearing blue on a red paper, while distilled water leaves the paper unaffected.

With blue paper No. 4 the attainment of equilibrium is very much more rapid because the paper is not glazed, and the rate of evaporation of ammonia is also accelerated though to nothing like the same extent. Blue paper No. 2 has a smaller "reaction inertia" than No. 1, besides it is already blue. It differs from No. 4 in that it is glazed. The instances cited above are exaggerated and, in the main, their interpretation obvious; but these factors operating in a minor degree may lead to incorrect conclusions.

Summary.

Except only in so far as the colour is influenced by the presence of neutral salts, the reaction of a solution is indicated by the colour of a piece of litmus paper which has been immersed in an excess of the fluid until no further change can be observed. The correction for neutral salt effect is a small one and is of the same order as that applying to litmus solution when used in the same way.

When the *quantity of solution used is limited in amount*, as when a drop of solution is placed on the paper and the effect observed, the following secondary influences determine the colour changes seen—

(1) Gradual attainment of reaction equilibrium between the litmus on the paper and the solution; more rapid with unglazed paper than with paper that is glazed.

(2) The actual effect of the reaction of the material in the paper on the

limited amount of solution used. This will, with the same paper, be most marked with a solution of lowest "reaction inertia." It is obviously fallacious to test the reaction of tap water by watching the effect of one drop of water on a heavily dyed paper.

(3) The "reaction inertia" of the paper. A thick heavily loaded paper has more effect in modifying the reaction of a drop or two of a solution than a thin paper lightly dyed with a litmus solution of low reaction inertia.

(4) The effect of exposure to the air. The evaporation or oxidation of some constituent of the solution affecting its reaction, will naturally produce a corresponding effect.

(5) The liberation of indicator from the paper, which is greater in the absence of neutral electrolytes and is more marked in alkaline than in acid solutions.

Determination of reaction of nearly neutral solutions of *proteins* and *their decomposition products* by means of litmus paper is very difficult. Such cases have not been dealt with fully here, but will form the subject of a later communication. The general effect is to indicate that the solution is more alkaline than it really is.

The same phenomena may be observed with litmus tincture to a much more marked degree. For this reason it has been frequently described as useless in such cases.

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XXVII. THE PREPARATION FROM ANIMAL TISSUES OF A SUBSTANCE WHICH CURES POLYNEURITIS IN BIRDS INDUCED BY DIETS OF POLISHED RICE.

PART I.

By EVELYN ASHLEY COOPER, *Beit Memorial Fellow.*

From the Lister Institute of Preventive Medicine.

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Although several workers have isolated from various food-stuffs substances capable in small amount of curing polyneuritis in pigeons and have ascribed chemical formulae to them, attempts to prepare the active constituent in an amount sufficient for investigating its chemical constitution and properties have up to the present time been unsuccessful.

Funk [1911, 1913] has isolated from rice polishings, yeast, milk, and ox-brain substances curative in oral doses of 0·02 g. and possessing similar melting-points.

Edie, Evans, Moore, Simpson, and Webster [1912] have also prepared a substance from yeast curative in small amount, to which they have ascribed the formula $C_7H_{17}O_5N_3$, and Suzuki, Shimamura, and Odaki [1912] isolated an active substance containing nitrogen from rice-polishings in the form of a picrate, but they have not published any results of its analysis.

1. Isolation of an anti-neuritic substance from horse-flesh.

In the present communication a method is described for preparing from horse-flesh a substance capable in minute amount of curing polyneuritis in pigeons.

The method employed was a modification of that described by Maclean [1912, 1] for the isolation and purification of the phosphatides of the kidney. The horse-flesh was minced, dried at 30° by means of an electric fan and ground, and the dry powder (weight 4000 g.) extracted at 37° with absolute alcohol on a shaking machine. The filtered extract was evaporated at 40° *in*

vacuo to remove the alcohol. The extract (weight 500 g.) was found to possess marked curative properties towards pigeons affected with polyneuritis, 4 g. being sufficient to bring about complete recovery within 24 hours. The extract was then treated with an excess of ether and the mixture allowed to stand in the cold room for 12 hours. The ether dissolved the fats and lipoids, but left undissolved a considerable amount of a white substance. This was washed thoroughly with ether and then tested on neuritic pigeons. Doses of 0·3 g. were sufficient to bring about complete recovery within 12 hours. This procedure therefore afforded a simple technique for separating a highly curative fraction from fats and lipoids and it was much more convenient than extracting the active substance by means of water.

The ether-soluble fraction was freed from ether by evaporating *in vacuo*. As much as 12 g. of the residue possessed only slight curative properties, while 2 g. of the lipoids separated from this fraction by means of acetone were inactive, but 7 g. were curative within 24 hours. It was thus possible to separate the bulk of the anti-neuritic substance from the alcoholic extract of horse-flesh by means of ether. These results support the explanation advanced by Maclean [1912, 2] for the curative properties of lecithin observed by several workers, namely, that the anti-neuritic substance is not a lipoid, but is present in ordinary lecithin as an impurity readily extractable therefrom by simple methods.

The ether-insoluble fraction was next treated with absolute alcohol, by which a large portion was dissolved. The insoluble residue contained both inorganic and organic material, but possessed no curative properties. The alcohol-soluble fraction however was strongly curative, and it was treated with excess of ether. This caused the separation of a yellow syrup (yield 50 g.) which was completely soluble in water and 0·2 g. of which was sufficient to cure pigeons affected with polyneuritis. The aqueous solution of the syrup was allowed to stand in a vacuum desiccator and a white crystalline substance gradually separated. This proved to be carnine and possessed no curative properties. The filtrate, which was highly curative, was next treated with finely powdered lead acetate, until there was no more separation of a flocculent precipitate, and the mixture was allowed to stand for 12 hours. The precipitate was filtered off, washed with water at 35°, decomposed with sulphuric acid, and the excess of acid removed by the careful addition of baryta. The filtered solution possessed no curative properties. The filtrate from the lead acetate precipitation was freed from lead by careful treatment with dilute sulphuric acid and was left very slightly acid. It possessed curative properties and was next treated with silver nitrate, which produced

a copious yellowish-white precipitate. This was filtered off, decomposed by hydrochloric acid and the resulting solution was nearly neutralised with soda and was found to be curative. The filtrate from the silver nitrate precipitation was also curative, but its content of active substance was completely precipitated by silver nitrate when baryta was added. By carrying out several animal experiments it was found that at least 3/5 of the total amount of anti-neuritic substance present in the filtrate from the lead acetate precipitation was precipitated by the addition of silver nitrate only, and about 1/4 was carried out of solution by the subsequent addition of baryta. The remaining 3/20 was probably destroyed by the alkali. The residue obtained by the evaporation of the curative solution resulting from the decomposition of the first silver nitrate precipitate with hydrochloric acid was next extracted with chloroform. Only a small amount of substance was extracted and this possessed no curative properties. The anti-neuritic substance was therefore insoluble in chloroform. It was also found to be insoluble in benzene and ethyl acetate. The residue insoluble in chloroform was next extracted with absolute alcohol; a large amount was insoluble, but the curative substance was found to have been entirely dissolved, and 0·10 g. of the soluble fraction was sufficient to cure a pigeon affected with polyneuritis. This fraction was then extracted with acetone and a considerable amount was dissolved. The insoluble residue, which contained all the active substance, was dissolved in 50 % alcohol, and the solution allowed to stand in a desiccator for some days. A white substance gradually crystallised out, 0·06 g. of which administered to each of two pigeons ameliorated the symptoms of polyneuritis in a few hours and effected a complete recovery within 24 hours. The substance dissolved with moderate readiness in water, but was insoluble in absolute alcohol.

It was not possible to obtain a sufficient amount of the active substance to determine its chemical composition, and the work is therefore being repeated on a much larger scale. As, however, it was found [Cooper, 1913] that cardiac muscle contained considerably more anti-neuritic substance than voluntary muscle, ox-heart has been substituted for the horse-flesh and the results so far obtained justify this change.

Some experiments have also been carried out to ascertain if the anti-neuritic substance could be precipitated by means of ether from the fats and lipoids derived from other animal-tissues. It was found that considerable amounts of curative fractions could be obtained by this procedure not only from horse-flesh and ox-heart, but also from horse-kidney, beef, ox-brain and liver.

Only a small amount of material, however, was separated by ether from the alcoholic extract of egg-yolk, and its curative power was very feeble. The results indicate that this fractionation affords quite a general method for separating the anti-neuritic substance from the fats and lipoids of animal tissues.

2. The Absorptive Capacity of Animal Charcoal for the anti-neuritic Substance.

Chamberlain and Vedder [1911, 1912] found that an extract of rice-polishings containing the anti-neuritic substance after being filtered through bone-black could no longer prevent polyneuritis in fowls and that the active substance could only be extracted to a small extent from the charcoal by water or alcohol. As these results are of great importance, the experiments have been repeated. Powdered animal charcoal was extracted three times with boiling water and then dried. A curative solution of known activity (prepared by dissolving the ether-insoluble fraction derived from an alcoholic extract of horse-flesh in water) was filtered slowly through a bed of the charcoal six times. The filtrate was still curative, but its activity was reduced to the extent of 30 %. Separate portions of the charcoal were then extracted with water and alcohol, and the extracts were found to be highly curative. The anti-neuritic substance was thus partially absorbed by charcoal and could be recovered from the latter by extraction with water or alcohol. It is seen that the results do not agree entirely with those obtained by Chamberlain and Vedder, but it is unlikely that the use of bone-black as an absorptive agent will be of much value for the isolation of the anti-neuritic substance.

3. The Effect of Sulphuretted Hydrogen upon the Active Substance.

In a preliminary fractionation of the horse-flesh extract sulphuretted hydrogen was employed to decompose the lead and silver precipitates and to remove the excess of the metals from solution, and during these processes a rapid disappearance of the active substance occurred. By substituting acids for the sulphuretted hydrogen (sulphuric acid for lead, hydrochloric acid for silver) it was possible to conduct the operations with a much smaller loss of active material.

These facts suggested that sulphuretted hydrogen destroyed the anti-neuritic substance. Accordingly, the effect upon activity of passing this gas

through a curative solution for four hours was investigated, but no evidence was obtained of any destruction of the anti-neuritic substance. The diminished activity during the above manipulations would therefore appear to have been due to the absorption of the curative substance by the colloidal metallic sulphides.

4. The Effect of Alkali upon the Active Substance.

Several investigators have observed that a considerable loss of anti-neuritic substance occurs when chemical operations involving the use of alkali are employed in the fractionations. It was thought desirable to throw some light on this matter by quantitatively studying the effect of treatment with alkali upon the curative power of the substance.

To a solution of known activity ammonia was added until its concentration throughout the total volume of liquid reached 10 %. The mixture was kept in a closed flask at ordinary temperatures for 24 hours, the ammonia then removed by a current of air, and the curative power of the solution redetermined. It was found that 50 % of the anti-neuritic substance had become destroyed by contact with the alkali.

These results indicate that the isolation of the anti-neuritic substance in large amount is only likely to be effected by employing chemical methods that do not involve the use of alkali and sulphuretted hydrogen.

5. The Effect of Alkaloids upon Pigeons affected with Polyneuritis.

Strychnine was found [Cooper, 1913] to prolong the lives of birds affected with polyneuritis but to exert no curative action. It was accordingly of interest to investigate the action of other alkaloids. Experiments have been carried out with quinine, cinchonine, and cinchonidine. The substances were dissolved in a trace of hydrochloric acid and administered to the birds orally.

Doses of quinine ranging from 0·01 to 0·1 g. exerted a temporary curative action. In six cases the symptoms were ameliorated for about three days and then became once more acute, further administration of quinine having no effect. In two other cases however the neuritic symptoms completely disappeared within a few hours, and one of the birds remained free from polyneuritis for four days, whilst the other, receiving daily in addition to polished rice 0·025 g. of quinine, remained healthy for 10 days and then died exhibiting

symptoms merely of weakness and not of acute polyneuritis. When given in amounts exceeding 0·1 g. however quinine had no ameliorative effect, but actually appeared to hasten the fatal issue.

Daily doses of quinine ranging from 0·01 to 0·025 g. administered to several pigeons fed on polished rice did not prevent or delay the development of neuritic systems.

Cinchonine in doses varying from 0·05 to 0·1 g. like quinine effected a marked improvement in the condition of pigeons suffering from polyneuritis, but within 48 hours the birds again exhibited the acute neuritic symptoms and died notwithstanding further doses of the alkaloid. Cinchonidine on the other hand had no ameliorative effect at all.

As quinine and cinchonine are extracted from cinchona-bark by means of acid, it was thought that their curative action might possibly be due to contamination with traces of the anti-neuritic substance present in the plants. The active substance is readily destroyed by heat, so that some light could be thrown on this matter by observing whether the alkaloids retained their curative properties after strong heating. It was actually found that after being heated at 125° for six hours quinine no longer had any ameliorative effect upon neuritic pigeons. As the alkaloid is not chemically altered by such treatment, it would appear that its curative action is due to the presence as an impurity of a minute amount of the anti-neuritic substance.

6. The Effect of Alcohol upon Birds fed on Polished Rice.

The administration of small doses of alcohol (0·5 c.c. three times daily) to pigeons fed on polished rice had no measurable effect upon the period of time elapsing before the occurrence of symptoms of polyneuritis, and thus appeared not to influence the utilisation of the supply of anti-neuritic substance distributed in the tissues of the birds.

This is presumptive evidence that alcoholic neuritis is not caused by any diminished capacity on the part of the organism to utilise the anti-neuritic substance which might be expected to result from the disturbing effects of alcohol upon the metabolism of the nervous system.

Summary.

1. A fraction rich in the anti-neuritic substance can be precipitated from the fats and lipoids (alcoholic extracts) of various animal tissues by means of ether.

2. A method based on this observation is described for isolating from horse-flesh a substance small amounts of which can cure polyneuritis in pigeons.

3. The substance is insoluble in absolute alcohol, benzene, chloroform, ether, and ethyl acetate, but is moderately soluble in water.

4. The substance is absorbed to some extent by animal charcoal and is readily destroyed by alkali. It is not inactivated by sulphuretted hydrogen, but disappears in large amounts during chemical operations in which colloidal metallic sulphides are formed.

5. Quinine and cinchonine exert a temporary curative action upon birds affected with polyneuritis. After being heated at 125° for 6 hours, however, quinine has no ameliorative effect, so that its curative properties would appear to be due to its contamination with traces of the anti-neuritic substance derived from the cinchona bark.

6. The administration of small doses of alcohol to birds fed on polished rice does not affect the period of time elapsing before the occurrence of polyneuritis, and thus appears not to influence the utilisation of the supply of anti-neuritic substance distributed in the tissues of the birds. This suggests that alcoholic neuritis does not result from a diminished capacity of the organism to utilise the anti-neuritic substance.

In conclusion I am glad to have this opportunity of expressing my indebtedness to Dr Hugh MacLean for drawing attention to the curative properties of the residues obtained by treating alcoholic extracts of voluntary muscle with ether and for much valuable help in the course of the investigation.

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XXVIII. ON THE LIPOLYTIC ACTION OF THE BLOOD.

By FRANCIS HUGO THIELE.

From the Research Laboratories, University College Hospital Medical School.

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Our knowledge of the ultimate processes involved in the storage of fat and its mode of transport from the fat depots to the tissues requiring it is very scanty. The fate of fat from the intestine to its presence in the thoracic duct is accurately known from the experiments of Moore [1897]. From the thoracic duct it passes into the blood and is rapidly converted into a substance no longer extractable by ether, which can pass through cell membranes to be stored up as fat in the fat depots or to be used as a source of energy. Of the nature of this substance our knowledge is practically nil. It has been shown by numerous observers that the tissues contain a "fat-splitting" ferment, the action of which under certain circumstances may also be reversible, and it was considered probable that the blood serum contained a similar ferment. This view was first of all advanced by Hanriot [1896], who tried the action of the blood on monobutyrin. His experiments, however, are erroneous as has been pointed out by Arthus [1902] because he took no precautions to ensure aseptic conditions and also because he used monobutyrin, which can be very easily hydrolysed, and it by no means follows that ferments, breaking up such simple esters as monobutyrin, have the same effect on neutral fats. Hagemeister [1903] showed that the injection of fat between muscle fibres was followed by its presence beneath the sarcolemma. He deduced that the lipolytic ferment of the tissue fluids caused the hydrolysis of the fat, diffusion of the resulting products occurring, followed by resynthesis in the muscle.

Fischler [1902, 1903] demonstrated the deposition of "fat" in organs after the injection of soap into the blood, or the perfusion of surviving organs with blood containing soap, thus lending support to the above. Munk [quoted by Fischler, 1902, p. 149], however, has shown that soap

even in small amounts exerts a toxic effect on the medullary centres. A small amount of soap apparently does occur in the blood and according to Schultz [1897] 28 % of the total fatty acids in the blood are in the form of soaps.

There is therefore no *a priori* objection to the possibility of the blood containing a lipase acting on the fats in the above-mentioned way and of the products being rapidly diffused into the cells to be resynthesised and stored up or used as required.

Experiments, however, conducted with strict precautions as to asepsis show that the blood serum does not contain a ferment capable of splitting ordinary fats.

The experiments of Cohnstein and Michaelis [1894] showed that there was a loss of ether-soluble substances on incubating mixtures of blood and chyle and a corresponding increase in water-soluble substances, and that this was due to a ferment in the erythrocytes. Their experiments have, however, the drawbacks that they were not absolutely aseptic and that there were inaccuracies in their mode of estimation.

Their results have been in part confirmed by Hamburger [1900] who was able to demonstrate a loss of ether-soluble substance after incubating a lipanin emulsion with blood.

Weigert [1900], however, considered that Cohnstein and Michaelis' results could be explained by autolysis of the lecithin. Hepner [1898] had previously shown that the blood plasma contains cholesterol and cholesterol esters of the fatty acids, and thought that the free cholesterol might conceivably have arisen by the action of a ferment on the cholesterol ester. Weigert therefore thought that the loss in ether extractives in Cohnstein and Michaelis' experiments could be accounted for by this, since the liberated fatty acids would become converted into soaps and thus no longer be ether-soluble. He experimented with blood plasma, blood and corpuscles alone. He found that blood plasma on incubation suffered no loss; when blood was incubated a loss of 18 % of ether extract occurred; whereas when corpuscles alone were thus treated there was a loss of 40–50 % of ether extract, and since Hepner and Hoppe-Seyler [quoted by Hepner, 1898] showed that red cells contained no cholesterol ester or neutral fat, the resulting change can only have been due to hydrolysis of the lecithin.

In the experiments of the author the following methods of extracting fat were adopted:

1. Drying the material with sand over a water-bath, and extracting with ether in a Soxhlet apparatus for 48 hours.

2. Mixing with anhydrous sodium sulphate as recommended by Pinkus, finely powdering the resulting dry mass, and extracting with ether at the room temperature for several days, with vigorous shaking at intervals. The extraction was repeated with fresh ether till nothing further was extracted.

3. Mixing with 94 % alcohol and keeping at 37° for several days, then filtering and repeatedly extracting the precipitate with alcohol, and finally boiling up. The united extracts were then evaporated and extracted with 94 % alcohol. The original residue was also extracted with ether, and the resulting extracts were divided into their constituents: fats, fatty acids and lecithin.

4. Digesting with pepsin and hydrochloric acid; evaporating with sand and extracting in the Soxhlet with ether.

Precautions to preserve absolute asepsis were taken in all these experiments. The various gases were allowed to bubble through the mixtures after having been filtered through sterile cotton wool. All specimens showing bacterial contamination, either by direct or cultural examination, were discarded.

I. Changes occurring when Blood alone is incubated.

Exp. 1.

100 c.c. of blood (by sand and ether extraction) yielded		0·403 g. fat
100 ,, ,, incubated at 37° for 24 hours in closed vessel yielded ...		0·381 ,,
100 ,, ,, same incubation, but with air current		0·297 ,,
Loss in specimen in air current = 26·3 %.		
,, without ,, = 5·4 %.		

Exp. 2.

100 c.c. of blood by sand and ether extraction yielded		0·366 g. fat.
100 ,, ,, incubated in air current for 24 hours at 37°		0·295 ,,
100 ,, ,, incubated without air at 37° for 24 hours		0·299 ,,
Loss in air current = 19·4 %.		
,, without ,, = 18·3 %.		

Exp. 3. Extraction after drying with anhydrous sodium sulphate.

(*a*) 100 c.c. of blood contained		0·24 g. fat.
100 ,, ,, after incubation in air current for 24 hours at 37° contained		0·184 ,,
Loss = 23·3 %.		
(*b*) 100 c.c. of blood contained		0·328 g. fat.
100 ,, ,, treated as in (*a*) contained		0·276 ,,
Loss = 15·9 %.		

For the purpose of determining to what the loss was due, the following estimations were made. The blood was extracted with alcohol and the residue with ether, the alcoholic extract was acidulated with dilute mineral

acid evaporated and shaken out with ether. The ether was freed from mineral acid by repeatedly shaking with water. The ethereal residues were united, evaporated, dried and weighed. The lecithin was determined by precipitation with acetone, the fatty acids by titration with N/10 alcoholic potash, and the neutral fat subsequently by boiling with a known quantity of N/10 absolute alcoholic potash.

250 c.c. of blood were taken.

	Unincubated blood		Incubated blood	
Total weight of residue	0·958 g.		0·936 g.	
Lecithin	0·28		0·127	
Fatty acids	0·152	(5·4 c.c. N/10 alc. pot.)	0·245	(8·7 c.c.)
Neutral fat	0·316	(11·2 „ „)	0·310	(11·0 c.c.)
Cholesterol	0·102		0·122	

With the exception of the increase in the cholesterol figure the whole difference is in the lecithin and fatty acids—the loss in the lecithin is 0·153 g., and the increase in the fatty acids is 0·093 g. Now if lecithin contains about 60 % fatty acids, then 0·153 g. contains 0·092 fatty acid.

Several other experiments showed a similar change.

II. Chyle Alone.

Exp. 1.

50 c.c. of chyle contained (by sand and ether method)	...	0·631 g. of fat.
50 „ „ incubated at 37° without current of air	...	0·579 „
100 „ „ incubated at 37° in air current ...	...	1·064 „

Loss without air current = 8·2 %.
„ in „ „ = 15·7 %.

Exp. 2. *Extracted by the Sodium Sulphate-Ether method.*

25 c.c. of chyle contained	...	0·317 g. of fat.
100 „ „ incubated in air current contained ...	...	1·178 „

Loss = 7·1 %.

Exp. 3. *Alcohol-ether extraction.*

100 c.c. of chyle	(*a*) Unincubated	(*b*) Incubated
Ethereal extract of the original extract	1·356 g.	1·243 g. (loss = 8·4 %)

After acidulating the residue of the original extract, shaking out with ether and removing mineral acid and adding the ethereal extracts together;

Lecithin found	0·056 g.	0·014 g.
Fatty acids	0·0	0·034
Neutral fat	1·208	1·137

From this it would appear that there was some hydrolysis of the lecithin and loss of a small amount of neutral fat. The fatty acid increase can be explained by the hydrolysis of the lecithin, since 0·048 g. lecithin contains about 0·029 g. of fatty acid.

III. Experiments on the Incubation of a Mixture of Chyle and Blood.

(1) The sand method of extraction.

Exp. 1.

100 c.c. of a mixture of equal parts of chyle and blood were found to contain 1·281 g. of fat.
100 c.c. of same mixture incubated for 24 hours without air current were found to contain ... ... 1·200 ,,
100 c.c. of same, incubated in an air current for 24 hours contained ... 0·9155 ,,
100 c.c. of same, incubated in an air current for 48 hours contained ... 0·8025 ,,

Loss was therefore:—in incubation without air current 6·4 %,
in 24 hours incubation with air current 28·5 %,
in 48 ,, ,, ,, ,, 37·3 %.

Exp. 2. Performed as above, showed;

100 c.c. of a blood chyle mixture contained ... ... 1·41 g. of fat.
100 c.c. of same incubated in air current at 37° for 24 hours ... ... 0·536 ,,
100 c.c. of same, incubated for 48 hours, same way ... ... 0·373 ,,

Loss after 24 hours was 61·9 %.
,, 48 ,, 73·5 %.

(2) Sodium Sulphate Method.

100 c.c. of a mixture of blood and chyle contained ... ... 1·228 g. of fat.
100 c.c. of the same mixture, incubated in a current of air at 48° for 24 hours 0·661 ,,

Loss = 46·2 %.

These figures show that on incubating chyle and blood together there is a large loss of ether extractive. This is not due to the method of extraction, it is as apparent with the one method as with the other. The loss is greater after air has been passed through the mixture, but contrary to the observation of Cohnstein and Michaelis [1894] it also occurs in the absence of an air current. The loss again is not the sum of the losses that would be obtained by incubating the blood and chyle separately.

Thus in the following experiments:

100 c.c. of blood yielded ... ... 0·29 g. of fat.
100 c.c. incubated for 24 hours with current of air at 37° yielded ... ... 0·232 ,,

Loss = 0·058 g. or 20 %.

100 c.c. of chyle yielded ... ... 1·202 ,,
100 c.c. incubated as above ... ... 1·034 ,,

Loss = 0·168 g. = 14·0 %.

Now a mixture of 50 c.c. of each contained ... ... 0·770 g. of fat.
But incubation as before yielded ... ... 0·478 ,,

Loss = 0·298 g. = 38·4 %.

Now the sum of the individual losses for 50 c.c. of each = 0·117 g., which is less than half the loss which occurred, when the two were incubated together.

This is also shown by the following experiment:

100 c.c. of chyle were found to contain	1·268 g. of fat.
100 ,, ,, yielded after incubation	1·168 ,,
Loss = 0·1 g.	
100 c.c. of blood yielded	0·314 ,,
100 ,, ,, after incubation yielded	0·244 ,,
Loss = 0·070 g.	
A mixture of 50 c.c. of chyle and 25 c.c. of blood were estimated to contain ...	0·712 ,,
After incubation a similar amount yielded	0·329 ,,

Loss = 0·38 g. = 53·8 %,

whereas the sum of the losses for each separate incubation

= 0·05 + 0·0175 = 0·0675 g.

The loss is thus considerably greater than the sum of the individual losses.

(3) To see if the serum contained the substance which caused this loss of substance extractable by ether a mixture was made of 100 c.c. of chyle and 100 c.c. of sheep's serum.

100 c.c. of the mixture contained	0·663 g. of fat.
100 ,, ,, ,, after incubation in an air stream after 24 hours yielded	0·591 ,,
Loss = 0·072 g. = 10·8 %.	

Similar experiments give like results up to 15 %.

(4) In order to determine whether the change is due to a ferment in the red corpuscles or other formed constituents, the following experiments were performed with carefully washed erythrocytes. These were obtained by centrifuging whipped blood and washing six times with sterile normal saline in sterile centrifuge tubes.

Exp. 1.

50 c.c. of chyle and 5 c.c. of red cells contained	0·802 g. of fat.
100 ,, ,, 10 ,, ,, incubated for 24 hours in an air current yielded	1·172 ,,
Loss = 0·43 g. = 26·9 %.	

In the next part of the experiment the chyle was first heated to 80° for 15 minutes:

100 c.c. of this with 10 c.c. of the suspension of red cells incubated for 24 hours at 37° yielded	1·107 g. of fat.

Loss for unboiled chyle was 26·9 %.
,, boiled ,, 30·9 %.

Exp. 2.

100 c.c. of chyle yielded	1·825 g. of fat.
20 c.c. of red corpuscles yielded	0·048 „
100 c.c. of chyle + 20 c.c. of the corpuscles incubated for 24 hours in an air current yielded	1·284 „

Loss was 31·4 %.

Exp. 3.

50 c.c. of chyle yielded	0·912 g. of fat.
20 c.c. of corpuscle suspension yielded	0·048 „

Both were heated to 80° and then incubated at 37° for 24 hours. The total amount of ether extract found was 0·937 g. The loss was therefore practically nil.

It will thus be seen that probably the red cells, or substances centrifugalised down with them, have the power of causing this loss of ether extractive.

(5) The next question investigated was whether this power was due to the haemoglobin, to a ferment in the cells, which may be to some extent diffusible into the plasma, or as stated above to elements precipitated by the centrifuge with the red corpuscles.

This was investigated by obtaining crystals of haemoglobin from dog's blood. The blood was whipped and centrifugalised, the corpuscles were then repeatedly washed with normal saline and then laked with ether, centrifugalised and washed with dilute alcohol and ammonium sulphate solution.

Exp. 1.

50 c.c. of chyle + 25 c.c. of a watery Hb solution yielded	0·550 ether ext.
Twice this amount was incubated at 48° for 24 hours in air current and yielded	0·729 „

Loss was 0·371 g.

Exp. 2. The haemoglobin was previously treated with a saturated solution of sodium fluoride in a saturated ammonium sulphate solution. The crystals were then repeatedly washed with a saturated solution of ammonium sulphate solution to remove the sodium fluoride.

50 c.c. chyle together with 50 c.c. watery Hb solution yielded	0·665 g. of fat.
The same amount after 24 hours incubation in a stream of air at 48° yielded...	0·632 „

Loss was 0·033 g. = 5 %.

Exp. 3.

100 c.c. of chyle was mixed with 50 c.c. of a watery solution of Hb. The Hb had been previously dried for several days at 50°. The mixture contained	1·268 g. of fat.
The same amount was incubated for 24 hours and yielded	1·202 „

Loss = 0·056 g. = 5·2 %.

To elucidate this point, whether the loss was due to the action of the haemoglobin, a mixture of chyle and blood was incubated and a mixed stream of carbonic oxide and oxygen gases was passed through the fluid. The carbonic oxide converted the haemoglobin into carboxy-haemoglobin and the oxygen was available if necessary.

By previous experimentation it was determined that the "lipase" obtained from pig's liver was unaffected by prolonged exposure to a stream of carbonic oxide gas as regards its action on ethyl butyrate.

The blood used was first saturated with carbonic oxide.

Exp. 1.

50 c.c. of blood and chyle yielded ... ... 0·490 g. of ether ext.
50 c.c. of the same mixture after incubation yielded ... ... 0·390 ,, ,,
Loss was 0·1 g. = 20·4 %.

Exp. 2.

100 c.c. of a mixture of equal parts of blood and chyle yielded ... 1·096 g. of ether ext.
The same amount after similar treatment yielded ... ... 0·731 ,, ,,
Loss was 0·365 g. = 33·3 %.

The experiments show that the change can still occur when the haemoglobin is put out of action as an oxygen carrier.

In these experiments it was certain that the haemoglobin was converted into carboxy-haemoglobin because of the colour of the mixture.

In the method of preparation of the haemoglobin crystals, proteins in the corpuscles or substances in the plasma like blood, dust particles, and leucocyte debris may be precipitated so that any possible ferment in connection with them would be precipitated as well. The action of the carbonic oxide and sodium fluoride leads to the supposition that there is a ferment separate from the haemoglobin.

(6) The following experiments were performed to see if the oxygen is really a necessity, or if the air current acts simply mechanically, thoroughly mixing the blood and chyle. The mixtures were incubated for 24 hours at 37°.

(*a*) 50 c.c. of a mixture of blood and chyle yielded ... ... 0·350 g. of fat.
100 c.c. of this mixture after treatment in coal gas yielded ... ... 0·542 ,,
Loss was therefore 0·158 g. = 22·6 %.

(*b*) 50 c.c. of blood and chyle contained ... ... 0·450 ,,
100 c.c. incubated in a stream of hydrogen gas contained ... ... 0·696 ,,
Loss was 0·204 g. = 22·5 %.

(*c*) 50 c.c. of blood and chyle mixture contained ... ... 0·395 ,,
100 c.c. of the same mixture in a hydrogen stream yielded ... ... 0·608 ,,
Loss was 0·182 g. = 23 %.

(*d*) 50 c.c. of chyle + 25 c.c. of blood contained ... ... 0·673 ,,
The same mixture incubated in air stream yielded ... ... 0·229 ,,
The same mixture incubated in CO_2 stream yielded ... ... 0·478 ,,
Loss in air = 0·444 = 66 %.
,, CO_2 = 0·195 = 29 %.

Thus it would appear that oxygen aids the action of the ferment, but on comparing with the result in (*c*) of this series it is obvious that mechanical agitation by the passage of a current of any gas also appreciably aids the process.

(7) The influence of protoplasmic and ferment poisons was next examined.

Exp. 1.

(*a*) 100 c.c. of a mixture of chyle and blood yielded ... ... 0·708 g. of fat.
100 c.c. of same mixture containing 5 % sodium fluoride was incubated for 24 hours in air stream at 48° and then contained ... ... 0·657 ,,
Loss was 0·051 g. = 7 %.

(*b*) 100 c.c. of the same mixture with potassium cyanide added and incubated in the same way yielded ... ... 0·641 ,,
Loss was 0·067 = 10 %.

(*c*) 200 c.c. of the same mixture was incubated in the ordinary way and yielded 0·547 ,,
Loss was 0·869 = 62 %.

(8) Some experiments were performed to determine the fate of the individual constituents.

Exp. 1. A mixture of equal parts of blood and chyle was made. (*a*) 100 c.c. were directly dried with sand and extracted with ether in the Soxhlet; (*b*) 100 c.c. were similarly treated after incubation in an air current at 37° for 24 hours.

	(*a*) Unincubated		(*b*) Incubated	
Total residue	0·732 g.		0·416 g.	
Neutral fats	0·583	(19·8 c.c.)	0·327	(11·1 c.c. N/10 alk.)
Fatty acid	0·0		0·0	
Lecithin	0·096		0·032	
Cholesterol	0·053		0·057	

There is thus, as was expected, a loss in neutral fat. The dried residue was then extracted with alcohol in the incubator at 37° while being continuously shaken, the alcoholic extract was evaporated and treated with mineral acid, and shaken out with ether, the ether subsequently being washed with water to remove the mineral acid. The ethereal extract was then titrated with N/10 alkali in alcohol.

The unincubated required 0·8 c.c. = 0·0226 g. fatty acid.

The incubated required 3·1 c.c. = ·0874 g. fatty acid.

On subsequently boiling the extract of the incubated specimen with a measured amount of N/10 alcoholic potash, it was found that 5·8 c.c. were used up = 0·1709 g. of fat, which had been liberated. This fat scarcely contained any trace of phosphorus. Hence the alcohol liberated a certain amount of neutral fat, which had become ether-insoluble to direct ethereal extraction.

Other experiments gave similar results as regards the amount of fatty acids present in the ether extract of the second alcoholic extraction, and showed that neutral fat was also present which did not come out in the original extraction.

Exp. 2. *A mixture of chyle and blood treated as in Exp.* 1.

	Non-incubated	Incubated
Total residue	0·746 g.	0·402 g.
Neutral fat	0·5938 (20·2 c.c. N/10 alk.)	0·294 (10 c.c.)
Fatty acid	0·0	0·0
Lecithin	0·99	0·029
Cholesterin	0·068	0·073
Subsequent extraction as above.		
Fatty acids	0·0328 g. (1·2 c.c.)	0·093 g. (3·3 c.c. alk.)
Fats	0·0	0·138 (4·7 „)

The same experiment was made with the same materials, but instead of subsequent alcoholic extraction the finely ground material after the ether extraction was digested with pepsin and hydrochloric acid and then dried and extracted with ether.

Exp. 3. Thus 100 c.c. of a mixture of blood and chyle were examined.

	(*a*) Unincubated	(*b*) Incubated
Primary extract	0·753 g.	0·449 g. (loss=43·1 %)
Secondary „	0·028	0·32
Fatty acid in secondary extract	trace	0·0789
Neutral fat	nil	0·233 (7·9 N/10 alkali)

The same method as above was used. 200 c.c. of a mixture of blood and chyle were used for immediate estimation by ether extraction followed by treatment with pepsin and extraction, and two separate lots of 200 c.c. were incubated in an air current at 37° for 24 hours, one examined by ether-alcohol extraction, and divided into its constituents, the other first extracted with ether and then digested with pepsin and acid and again extracted with ether.

Exp. 4.

	Control	Specimen "A" ether-alcohol extraction	Specimen "B" digestion with pepsin, etc.
	g.	g.	g.
Total residue (first extract)	1·64	0·813 (loss=50·4 %)	0·799 (51·8 %)
Neutral fats	1·3934 (47·2 c.c. N/10 alk.)	0·705 (23·9 c.c. N/10 alk.)	—
Fatty acids	0·0	0·0	—
Lecithin	0·198	0·087	—
Cholesterol etc. ...	0·0486	0·021	—
Fatty acid in second extract	0·0479 (1·7 c.c. N/10 alk.)	0·121 (4·3 c.c. N/10 alk.)	0·1325 (4·7 c.c.)
Fat in second extract ...	nil	0·545 (18·5 c.c.)	0·7139 (24·2 c.c.)

It was also found that by extracting with alcohol on the water-bath and then with ether, the whole of the ether-insoluble fat could be recovered.

Two lots of 100 c.c. of a mixture of chyle and blood were examined (*a*) unincubated, (*b*) incubated in the usual way.

	(*a*) Unincubated	(*b*) Incubated
Primary extract	0·712 g.	0·467 g.
Secondary „	0·028	0·298

These experiments further show that the neutral fats are not hydrolysed by the blood or chyle, but that the lecithin is the only fat hydrolysed by the ferments in the chyle or blood. This is at variance with the observation of Hamill [1907] who states that the chyle can hydrolyse a neutral fat, viz., olive oil.

(9) The question then arose if this ether-insoluble fat could be isolated and its constitution determined and especially if it were diffusible and soluble in water.

The method adopted was that of Cohnstein and Michaelis [1894], viz. diffusion through parchment thimbles into distilled water. In all the experiments it was found that only soaps, etc., diffused through, and no substance containing the neutral fat, and that the amount of fatty acid thus obtained corresponded to the degree of hydrolysis of the lecithins.

Experiments were also made with a lipanin emulsion, made with sterile ascitic fluid and lipanin. A very fine emulsion was produced.

It was found that on incubation with blood and a current of air the same results were obtained as above with chyle fat and that the fat could be recovered again by treatment with alcohol or pepsin and hydrochloric acid.

These observations are in accordance with those of Mansfeld [1907] who showed that the fat in lipanin after incubation with blood became non-extractable by ether, but could be recovered by Liebermann's method.

Hamburger [1900] was only able to obtain this result when he made his artificial chyle with a chyloid fluid. In these experiments, as in those of Mansfeld, ordinary ascitic fluid worked well.

Conclusions.

It would seem justifiable to draw the following conclusions:

1. Blood and chyle contain a ferment which can hydrolyse lecithin but not neutral fat.

2. When blood and chyle fat are incubated together, the neutral fat forms an absorption combination with the proteins and is thus rendered non-extractable by ether. The combination can, however, be broken up by peptic digestion, or treatment with alcohol. It is not formed during the process of drying.

3. The combination appears to be broken up by heat, since larger losses are obtained if the incubated mixtures are not heated, and if the material be dried at a low temperature.

4. The formation of this complex appears to be due to the formed elements of the blood, since the corpuscles or material carried down with the corpuscles on centrifugalisation have the same effect, and serum by itself has not.

5. The action does not appear to be due to the haemoglobin, but to some material precipitated in the method of preparing the haemoglobin, since on rendering the haemoglobin inert as an oxygen carrier by carbon monoxide, the change still occurs.

6. The change occurs best in a current of air or oxygen, and if the mixture be thoroughly agitated.

7. The combination appears to be due to the action of a ferment because:

(*a*) the haemoglobin may be rendered inert without affecting the process;

(*b*) ferment poisons seriously interfere with it.

8. Similar combinations may occur when blood is mixed with a very fine emulsion of fat. The compound, however, does not appear so stable since it is practically always broken up by heating and by alcohol.

9. The albumin-fat combination is not diffusible nor soluble in water. After incubation an increase in diffusible material takes place, but this is only due to the diffusion of the products of the lecithin hydrolysis, viz., glycerophosphoric acids, soaps, choline.

10. The chyle and corpuscles contain a ferment capable of breaking up lecithin and liberating the fatty acids.

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XXIX. ON THE LIPOLYTIC ACTION OF THE TISSUES.

By FRANCIS HUGO THIELE.

From the Research Laboratories, University College Hospital Medical School.

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The following experiments were carried out to test the lipolytic activity of the various organs towards the neutral fats.

It is only comparatively recently that such enzymes have been demonstrated in the tissues; their presence was inferred previously from the action of tissue extracts on simple esters, such as monobutyrin, etc. That these are by no means the same, has been shown by Cohnstein [1904] for the castor-oil seed. Arthus [1902] showed a similar difference for blood serum.

Nencki and Lüdy [1887] tested the lipolytic power of tissues on amyl salicylate and found that the tissue extracts worked best in an alkaline medium.

Hanriot [1896] used monobutyrin and demonstrated a lipolytic ferment in all tissue extracts except the thyroid. The enzyme ceased to act when a certain degree of acidity had developed and could continue when the acidity was neutralised. He also showed that the tissue extracts could hydrolyse the ethyl esters of formic, acetic, propionic and butyric acids.

Arthus [1902] drew attention to the errors in Hanriot's observations and was unable to confirm his work. Kastle and Loevenhart [1900] experimented with ethyl butyrate and found that the ferment was closely attached to the cells, and that liver extract was more active than the pancreatic on this ester, but that the pancreas could hydrolyse the esters of the higher fatty acids more readily than those of the lower.

Siebert [1900] studied the action of pancreatic extracts on egg yolk, and further, showed that the blood serum is incapable of splitting lecithin. Subsequently Umber and Brugsch [1906] experimented with tissue extracts, using egg yolk as their emulsion of neutral fat. The various organs were washed free from blood, then finely ground up with kieselguhr and the juice

pressed out with a hydraulic press. Two series of experiments were performed, one with the organs from an animal killed whilst digesting, the other in starvation. One series of experiments was conducted under aseptic conditions, the other under toluene.

These observers concluded that the tissue extracts can hydrolyse the fat in egg yolk and that during digestion the pancreatic extract is the most powerful, closely followed by that of the spleen, but that the liver and intestinal mucosa are more powerful than the pancreas. Further that activating bodies would appear to be present in some of the organs.

In the following series of experiments it was intended to study the action of the various tissue extracts on:

(1) Egg yolk.
(2) Artificial emulsions of lecithin.
(3) Lecithin not in a state of emulsion.
(4) Chyle obtained from the human subject.

The tissues were ground up finely in a mortar, mixed with an equal bulk of normal saline and pressed through a press so that the tissue extract consisted of a fine suspension of the tissue in normal saline. The tissues were removed under strict aseptic precautions; pigs' and dogs' organs were chiefly used. In the case of the pigs' organs, these were removed in the slaughter-house in gauze soaked in 2·5 % carbolic, the outside was removed with a sterile knife and the tissue transferred to a sterile mortar and ground up with sterile normal saline. The dogs' tissues were obtained from healthy dogs, which were anaesthetised and bled to death. The animals were subsequently washed out with sterile normal saline. No toluene experiments are recorded, because it was found that even adding toluene up to 2 % did not inhibit the growth of organisms which were capable of splitting up egg yolk very readily. The "fat" containing emulsions were also prepared under strictly aseptic precautions and sterilised.

The mixtures of "fat" and tissue extract were incubated at 37° for 15–20 hours. At the end of that time they were examined directly and culturally for possible bacterial contamination and any showing this were rejected. The method of estimating the effect of the tissue extracts was to determine the total amount of "fat" present by boiling the mixture of unincubated material with sodium carbonate for some time on the water bath, and then, after acidulating with sulphuric acid, extracting with ether. The ethereal extract, after removal of the mineral acid, was then treated with N/10 KOH to neutralise the fatty acids, and then hydrolysed with alcoholic potash, evaporated, and taken up in water, acidulated with sulphuric acid

and the liberated fatty acids extracted with ether, which was then freed from sulphuric acid by shaking up with water. The ethereal solution of the fatty acids was titrated against N/10 alcoholic potash and then reckoned in terms of oleic acid; 1 c.c. N/10 KOH = 0·0282 g. oleic acid. The incubated mixtures were neutralised with sodium carbonate, well heated on the water bath and acidulated with sulphuric acid to liberate the fatty acids which were extracted with ether and purified as before. The amount of fatty acid was thus determined by titration with N/10 alcoholic potash.

In some of the experiments it became necessary to separate oleic acid and other high fatty acids from any lower acids, such as lactic acid, which might be formed from the carbohydrates. This was done by having a double series of mixtures; in the one the total fatty acids were determined in the way mentioned above, in the second the mixture, after neutralisation and boiling, was treated with sulphuric acid to liberate the fatty acids and then treated with ammonium sulphate, according to the methods of Magnus Levy, to separate the lower from the higher fatty acids.

A. *Effect of tissue extracts of various organs on sterile egg yolk mixture, containing 0·5 % sodium carbonate.*

10 c.c. tissue extract was used with 20 c.c. of yolk suspension. Results expressed in c.c. decinormal alkali.

No. of Exp.	Pigs' organs	Total fats as fatty acids	Fatty acids liberated	% hydrolysed
1	Pancreas	53·2	48·0	90·0
	Liver	55·8	20·5	36·7
	Spleen	53·8	20·2	37·5
	Pancreas + 5 c.c. Spleen ...	53·4	40·0	75·0
	,, ,, ,, (the spleen was first boiled) ...	53·4	40·0	75·0
	Liver + 5 c.c. Spleen	56·0	21·6	38·6
	Pancreas + 5 c.c. Spleen ...	54·2	38·6	70·1
	Serum	Not determined	0·0	0·0
2	Pancreas	53·6	30·6	57·0
	10 c.c. Bile	51·8	19·08	36·7
	Pancreas + 5 c.c. Bile	54·0	30·0	55·5
	Pancreas + 5 c.c. Bile + 5 c.c. Serum	54·6	30·8	56·0
	10 c.c. Serum	Not determined	0·0	0·0
3	Pancreas	54·0	37·2	68·2
	Liver	55·2	21·8	39·5
	Kidney	54·6	20·4	37·87
	Muscle	54·4	20·2	37·0
	Spleen	54·4	21·6	40·0
	Pancreas + 5 c.c. Spleen ...	54·8	39·1	71·0
	Muscle + 5 c.c. Spleen ...	54·4	21·0	39·87

Dogs' Tissues.

5 c.c. of extract + 20 c.c. of yolk suspension in each experiment. Results expressed as before.

		Total fats	Fatty acids	% hydrolysed
Dog 1 Hungry animal	Spleen	56·1	20·5	36·5
	Liver	56·8	20·5	36·0
	Pancreas	55·9	22·8	40·9
	Kidney	56·3	20·2	35·8
	Liver + Spleen (5 c.c. of each) ...	57·1	22·0	38·0
	Pancreas + Spleen (5 c.c. of each)	56·2	37·0	65·8
	Pancreas + 5 c.c. of Serum ...	58·4	37·5	66·2

5 c.c. of extract + 20 c.c. of yolk suspension in each experiment. Results expressed as before.

Dog 2 Digesting animal	Pancreas	54·2	41·0	75·5
	Pancreas + Serum	54·4	37·0	68·0
	Serum	—	0·0	0·0
	Liver	55·0	20·8	37·8
	Spleen	54·3	20·2	37·0
	Pancreas + Spleen	54·6	44·5	81·5
	Pancreas + boiled Spleen ...	54·6	41·5	77·0

5 c.c. of extract + 20 c.c. of yolk suspension in each experiment. Results expressed as before.

Dog 3 Hungry animal	Pancreas	52·8	30·0	57·0
	Pancreas + Liver (5 c.c. of each)	53·6	34·0	63·4
	Pancreas + Spleen (5 c.c. of each)	53·2	33·8	61·2
	Spleen	53·9	21·2	40·0
	Liver	53·4	22·0	40·1

Human Tissues.

Removed twelve hours after death. 20 c.c. of egg yolk and 10 c.c. of the tissue suspension. Results expressed as before.

	Total fats as fatty acids	Fatty acids liberated	% liberated
Pancreas	56·2	46·0	81·8
Pancreas and Liver (5 c.c.) ...	57·0	48·0	84·3
Pancreas and boiled Liver (5 c.c.)	57·0	49·0	85·9
Liver	56·8	21·0	37·0
Spleen	56·0	21·4	38·2
Liver + 5 c.c. Spleen	57·0	23·0	40·3
Pancreas + 5 c.c. Spleen... ...	56·6	48·6	85·6

Sheep's Tissues. Quantities as before.

Liver	54·8	20·2	36·6
Spleen	54·0	19·8	37·0
Liver + Spleen (5 c.c.)	55·0	20·4	37·0
Liver + boiled Spleen (5 c.c.) ...	55·2	20·4	37·0
Pancreas	53·8	30·2	56·4
Pancreas + 5 c.c. Spleen... ...	54·2	32·0	58·6

From these experiments it will be seen that the tissues have the power of breaking up the lecithin when it is exhibited to them in the form of egg yolk emulsion.

In all cases the pancreas has a much more powerful action than any other tissue extract, and there is from these experiments no distinct evidence of

any kinase capable of augmenting the activity of the ferments. The only example of this effect occurs in one of the experiments with the tissues of a dog in a fasting state, but in the other experiments with the organs of a dog, and with those of pigs and sheep, which are always slaughtered in a fasting condition, there is absolutely no evidence of any activating ferment in any of the tissues. In these respects these experiments do not agree with those of Umber and Brugsch [1906].

The above results, however, like those of Umber and Brugsch, take no notice of the formation of the lower fatty acids, which Magnus Levy [1902] showed were formed during the incubation of tissues from the carbohydrates present. In order, therefore, to eliminate this error, the following experiments were performed in double series; in one the total fatty acids were estimated, in the other the lower fatty acids were estimated after separation by ammonium sulphate, the difference between the two being due to the amount of fatty acid liberated by the hydrolysis of the fat.

In the following series of experiments 10 c.c. of tissue juice were incubated with 20 c.c. of yolk suspension in 0·5 % sodium carbonate at 37° for 18 hours. The results here are in terms of N/10 NaOH as before.

No. of Exp.	Pigs'	Total fatty acids found	Lower fatty acids	Higher fatty acids	Total fat in terms of fatty acids	% hydrolysed
1	Liver	23·0 c.c.	7·6 c.c.	15·4 c.c.	52·0	29·6
	Spleen	21·0	3·2	17·8	51·2	34·7
	Kidney	18·0	4·0	14·0	51·3	27·3
	Pancreas	39·0	1·5	37·5	51·0	73·5
2	Sheep's					
	Pancreas	31·0 c.c.	2·0 c.c.	29·0 c.c.	51·0	56·8
	Liver	19·0	6·1	12·9	51·8	27·9
	Spleen	12·8	1·8	11·0	51·2	21·5
	Kidney	13·2	4·0	9·2	51·4	17·9
3	Dogs'					
	Pancreas	38·0 c.c.	1·4 c.c.	36·6 c.c.	49·2	74·4
	Liver	18·0	4·1	13·9	49·8	27·8
	Spleen	17·0	5·7	11·3	49·0	23·0
	Muscle	12·0	5·2	6·8	49·3	13·7

These experiments do not very greatly alter the results of the previous experiments, and confirm the lipolytic power of the various tissue extracts towards egg yolk emulsion.

B. *Experiments with liver extracts on lecithin, not in a state of emulsion.*

Weighed quantities of lecithin were placed in sterile flasks and sterilised. Then sterile tissue extract was added and allowed to act for varying intervals in the cold or warm.

In Exp. 1.	19 % was broken up.	}	In 16 hours at 37°.
,, 2.	23 % ,, ,,		
,, 3.	18 % ,, ,,		In 14 days at 4°.
,, 4.	56 % ,, ,,		,, 28 ,, ,,

The result in each case should be somewhat higher, because no account was taken of the amount of lecithin in the liver extract added. It is interesting to note that jecorin could also be found in all these experiments after incubation.

C. *Action of tissue extracts on chyle.*

The chyle was obtained from a healthy young man suffering from lymphatic obstruction, producing lymphangiectasis in one lower limb. The chyle could be obtained under aseptic conditions in large quantities after a period of blocking the fistulae in the lower limb. It contained from 1·3 to 1·6 g. of fat per cent., and from 0·064 to 0·08 g. of lecithin.

The lipolytic ferment in the chyle was destroyed by heating to 60° for an hour, thus also sterilising the fluid.

Sodium carbonate was added to make a 0·5 % solution.

In these experiments a double series was made in order to determine the relative amounts of higher and lower fatty acids.

20 c.c. of extract of the finely pounded tissue were added to 50 c.c. of chyle and incubated for 18 hours at 37°. Results expressed in c.c. of N/10 NaOH.

No. of Exp.	Pigs'	Total fats	Fatty acids found	Lower fatty acids	Higher fatty acids	% hydrolysed
1	Pancreas	24·0	18·8	1·6	17·2	71·5
	Liver	27·2	13·0	6·4	6·6	24·2
	Spleen	23·1	5·6	2·6	3·0	13·0
	Kidney	24·8	9·4	4·2	5·2	21·0
2	Pigs'					
	Pancreas	22·9	14·2	0·8	13·4	58·5
	Liver	25·8	9·2	3·8	5·4	20·9
	Spleen	23·2	7·4	4·8	2·6	11·2
	Kidney	24·4	6·8	3·0	3·8	15·6
3	Sheep's					
	Pancreas	22·0	13·2	1·0	12·2	55·4
	Liver	27·1	9·2	4·0	5·2	18·0
	Spleen	22·4	5·6	3·2	2·4	10·7
	Kidney	25·0	6·0	2·0	4·0	16·0
4	Dogs'					
	Pancreas(10c.c.)	21·6	15·0	1·0	14·0	65·0
	Liver	30·3	13·4	4·2	9·2	30·3
	Spleen	24·7	6·5	2·8	3·7	15·0
	Kidney	26·6	8·2	3·1	5·1	20·0

These results show that a certain amount of the fatty acids found in the

incubated mixture is due to the formation of lower fatty acids. Further, that the tissues have the power of liberating higher fatty acids when incubated with chyle, but as chyle and the tissues contain not only neutral fat, but lecithin as well, the question then arises as to the relative proportion hydrolysed by the tissue ferments. Finally the figures are quite small when compared with those obtained by using egg yolk.

To determine this question a series of experiments was carried out in which the lecithin, the total amount of fats and the higher fatty acids formed were determined before and after incubation.

For this purpose three sets of each mixture were made. The lecithin was determined by extracting the material with alcohol and ether, evaporating, taking up in ether, precipitating by acetone and determining the phosphorus in the precipitate by Neumann's method.

1 c.c. N/2 NaOH = 0·014331 g. of lecithin.

Lecithin contains 66 % fatty acids.

Quantities as before. Results reckoned as fatty acids in terms of N/10 alkali.

Exp. 1. Pigs'	Liver	Pancreas	Spleen
Total fats	25·1 c.c.	21·1 c.c.	23·5 c.c.
Lecithin	0·261 g.	0·0902 g.	0·1504 g.
Lecithin fatty acids ...	6·1 c.c.	2·1 c.c.	3·5 c.c.
Fatty acids liberated ...	10·1	14·0	6·8
Lower fatty acids liberated	4·2	1·8	3·0
Higher ,, ,,	5·9	12·2	3·8
Lecithin left	nil	nil	nil
Fatty acid in Lecithin ...	6·1	2·1	3·5
Fatty acid found ...	5·9	12·2	3·8

Exp. 2. Dog's Liver. Quantities as before.

Total fats	25·9 c.c.
Lecithin	0·339 g.
Lecithin fatty acids	7·9 c.c.
Fatty acids liberated	12·2
Lower fatty acids liberated	4·0
Higher ,, ,, ...	8·2
Lecithin left	nil

Fatty acids in the lecithin = 7·9 c.c. N/10 NaOH.
,, ,, found liberated = 8·2 ,, ,,

Exp. 3. Quantities as before.

Sheep's	Liver	Spleen	Kidney
Total fats as fatty acids ...	30·4 c.c.	28·9 c.c.	30·2 c.c.
Lecithin	0·244 g.	0·177 g.	0·235 g.
Fatty acids in Lecithins ...	5·7 c.c.	4·2 c.c.	5·5 c.c.
Fatty acids liberated ...	11·3	8·4	7·8
Higher fatty acids liberated	5·5	3·9	5·3
Lower ,, ,,	5·8	4·5	2·5
Lecithin left ,,	nil	nil	nil

Exp. 4. Pigs'	Liver	Kidney	Spleen	Spleen + Liver (10 c.c. of each)
Total fats	26·8 c.c.	25·4 c.c.	24·2 c.c.	21·1 c.c.
Lecithin	0·2178 g.	0·1576 g.	0·1089 g.	0·3152 g.
Fatty acid in lecithin ...	5·1 c.c.	3·7 c.c.	2·6 c.c.	7·4 c.c.
Fatty acids liberated ...	8·3	5·2	6·5	13·7
Lower fatty acids liberated	2·8	2·1	4·0	6·1
Higher ,, ,,	5·5	3·1	2·5	7·6
Lecithin	nil	trace	nil	nil

In all these experiments it will be noticed, with the exception of that with pancreatic extract, that the amount of fatty acids liberated corresponds to the amount of fatty acids present in the lecithin, and that in all cases the lecithin is practically completely hydrolysed.

Evidence of the inability of the lipase of the liver to attack neutral fat is obtained from experiments on aseptic autolysis at 4°. Fat expressed as c.c. N/10 NaOH %.

Duration of autolysis (days)	Dogs' Tissues	Sheep's Tissues
0	16·2	21·0
7	16·6	20·4
21	15·8	21·6
42	16·2	22·4
91	16·8	—
168	—	22·8
224	—	22·4

This shows that ordinary fat is not hydrolysed, but that lecithin is hydrolysed at 4°. These results indicate that the liver, spleen and kidney contain a lipolytic ferment which attacks lecithin quite readily, but has no apparent action on the simple glycerides of the higher fatty acids even when presented in the form of the finest emulsion as in chyle, or as they exist in the tissue itself as is seen in the autolytic experiments.

It is possible that the reason of this lies in the fact that lecithin can form in water a colloid suspension, and so can be more readily attacked, but the previous results show that lecithin can be readily split up even when presented to the tissue extracts in a very coarse form. The other and more probable explanation would appear to be that when the glycerides of the higher fatty acids are taken to the liver or other tissues they are converted into lecithins and stored up free or in combination with the protein as the invisible unstainable fat in the normal organ and in this form are easily hydrolysed by the lipolytic ferment as required. This view seems the more probable from the results of Leathes and Kennaway [1909], who showed that the liver desaturates fats brought from the food or fat depots; this agrees with the high iodine values of the lecithins, showing that the lecithins are

formed from these desaturated fatty acids. Further, it is important to note in the quantitative estimations of the "fats" in the liver, etc., that by far the greatest amount is accounted for by the lecithins, nearly 90%.

D. *Experiments to ascertain the effect of reaction on the lipolytic power of the organs.*

In each of these a double series of experiments was performed to ascertain the amount of higher fatty acids.

Pig's Liver. 10 c.c. of extract with 20 c.c. of egg yolk suspension.

1. *Effect of Alkali.*

With g. Na_2CO_3	(%)	Total fatty acids	Lower	Higher	Total fat	% hydrolysed
0·05	(0·16)	14·4	6·0	8·4	40·8	20·7
0·1	(0·32)	21·6	7·6	14·0	40·8	34·3
0·15	(0·5)	23·2	7·4	15·8	40·8	38·7
0·2	(0·66)	18·2	6·1	12·1	40·8	29·6
0·3	(1·0)	10·1	4·0	6·1	,,	14·9
0·4	(1·33)	6·0	2·1	3·9	,,	9·0
Same quantities as above, but 30 c.c. water added.						
0·05	(0·08)	12·5	4·6	7·9	40·2	19·6
0·1	(0·16)	20·0	6·0	14·0	,,	34·8
0·15	(0·24)	21·4	6·6	14·8	,,	36·8
0·2	(0·33)	16·2	4·6	11·8	,,	29·3
0·3	(0·5)	9·3	2·4	5·9	,,	14·6
0·4	(0·66)	5·0	2·0	5·0	,,	7·4

Thus it will be seen that the activity does not depend on the percentage of alkali present, but on the total amount.

2. *Effect of lactic acid.* The quantities used were as before. Incubation 16 hours. The amount of lactic acid is in c.c. of the decinormal solution.

Dog's Liver. 10 c.c. of extract with 20 c.c. of egg yolk solution and all made up to 40 c.c.

C.c. N/10 acid	Total fats c.c. N/10 alk	Total fatty acids found	Lower	Higher	% hydrolysed
2	48·2	20·2	6·8	13·4	27·8
4	,,	23·8	7·9	15·9	32·9
6	,,	24·9	9·3	15·6	32·6
8	,,	23·1	12·4	10·7	22·2
10	,,	20·8	13·0	7·8	16·2
Pig's Liver. Quantities as above.					
2	48·2	18·6	8·1	10·5	21·8
4	,,	23·8	9·7	14·1	29·2
6	,,	24·2	10·4	9·8	20·3
8	,,	21·2	11·8	9·4	19·5
10	,,	19·8	14·1	5·7	11·8

SUMMARY.

From these experiments we can conclude that:

(1) The tissues possess a true lipolytic ferment.

(2) The lipolytic ferment, with the exception of the pancreatic lipolytic ferment, can only hydrolyse phosphatides and jecorins, but not ordinary fats.

(3) The ferment is capable of acting in an alkaline or acid medium.

(4) There is no evidence of a kinase in the spleen.

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XXX. THE ESTIMATION OF TYROSINE IN PROTEINS BY BROMINATION.

By ROBERT HENRY ADERS PLIMMER and ELIZABETH COWPER EAVES.

From the Institute of Physiology, University College, London, and the Physiological Laboratory, University of Sheffield.

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The tyrosine content of a protein is usually ascertained by hydrolysing the protein with sulphuric acid, removing the acid as barium sulphate, concentrating the solution and washings and then weighing the tyrosine which separates out. The data so obtained by various workers are in some cases very concordant, but it is generally considered that the real tyrosine content of a protein is higher than these figures represent since it is frequently impossible to isolate the whole of the tyrosine. Osborne and Clapp [1906] and Osborne and Guest [1911] have particularly emphasised this point and they consider that the tyrosine content of a protein so obtained is only a minimal one, the true content being from one half to one per cent. higher. The presence of cystine in the tyrosine isolated has also never been taken into consideration. The two substances are so alike in their solubility in water, in acids and in alkalies that their separation is a matter of considerable difficulty [Plimmer, 1913]. Losses occurring in the isolation of the tyrosine may be compensated for by admixture with cystine.

It was shown by J. H. Millar [1903] that tyrosine was readily brominated and converted into dibromotyrosine, and that the amount of tyrosine in a simple mixture of amino-acids could be accurately estimated by means of this reaction.

A. J. Brown and E. T. Millar [1906] using this reaction showed that the tyrosine was completely liberated at a very early stage in the hydrolysis of edestin by trypsin.. Their data gave the tyrosine content of edestin as 4·06 per cent., a figure which is considerably higher than that obtained by direct isolation (2·1 per cent.). They made no estimations of the tyrosine

content of other proteins. The higher value agrees with Osborne's supposition that the tyrosine content of proteins as obtained by isolation is only a minimal one and it seemed very desirable that further estimations should be made by this method.

It was found that it was better to alter Millar's method as it was not sufficiently delicate for estimating small amounts of tyrosine such as are obtained by the hydrolysis of proteins. The procedure of Brown and Millar also involved a large deduction for the amount of bromine absorbed by the control (protein or other decomposition products, possibly histidine) which prevented an accurate estimation. The absorption of bromine by the unchanged protein and its product of hydrolysis, histidine, which according to Knoop [1908] reacts with bromine, has been eliminated by the use of phosphotungstic acid. Another disturbing factor is the presence of tryptophane with the tyrosine amongst the products of hydrolysis. Though tryptophane may be destroyed by boiling with acids, its decomposition products still absorb bromine. The estimation of tyrosine amongst the products of the acid hydrolysis of proteins was therefore impossible. It remained to take advantage of the rapid and complete liberation of tyrosine during the early stages of tryptic digestion and the slower liberation of tryptophane, which is only complete after several days when a moderately active trypsin solution is employed [Hopkins and Cole, 1901]. By estimating the bromine absorption after short intervals of digestion in the filtrate from the phosphotungstic acid precipitate, values for the tyrosine content of proteins have been obtained which agree closely with those found by direct isolation.

Our work was withheld since we felt considerable diffidence as to the exactness of our data, but since the publication of much higher figures for the tyrosine content of proteins by Folin and Denis [1912] by a new colorimetric method and since these figures have been criticised by Abderhalden and Fuchs [1913] our data are of some value in support of the results of Abderhalden and Fuchs. Folin and Denis do not seem to have taken sufficient account of the presence of tryptophane and oxytryptophane, which react with their reagent. If their figures express the total tryptophane and tyrosine content, and our values the tyrosine content alone, then the difference will give the tryptophane content of proteins which is at present unknown and of considerable importance.

Experimental.

(1) *The Estimation of Small Amounts of Pure Tyrosine by Bromination.*

J. H. Millar's method for the estimation of tyrosine depends upon the formation of dibromotyrosine when tyrosine is treated with nascent bromine; this is liberated by adding sodium bromate to an acid solution of potassium bromide. The equations representing the reaction are:

$$NaBrO_3 + 5KBr + 6HCl = NaCl + 5KCl + 3Br_2 + 3H_2O,$$

$$C_6H_4(OH).CH_2.CH(NH_2).COOH + 2Br_2 = C_6H_2Br_2(OH).CH_2.CH(NH_2).COOH + 2HBr,$$

from which we find that 1 g. of tyrosine absorbs 1·765 g. of bromine and corresponds to 0·5558 g. of sodium bromate.

His procedure was to add 10–15 cc. of a 20 per cent. solution of potassium bromide to a solution of tyrosine in hydrochloric acid and to titrate with M/5 sodium bromate solution until it assumed a persistent deep yellow colour. If the solution were coloured, starch and potassium iodide were used as an indicator.

Millar found that 1·808 g. of bromine were absorbed by 1 g. of tyrosine, a figure which is slightly higher than the theoretical value but of sufficient accuracy to show that tyrosine can be estimated in solution by bromination; in one of the experiments 0·2 g. tyrosine required 3·8 cc. of the bromate solution.

The estimation of smaller amounts of tyrosine than 0·2 g. was not investigated by J. H. Millar. The amount of tyrosine in a protein does not usually exceed 3 per cent. except in the case of caseinogen which contains from 4·5–5 per cent. and silk-fibroin which contains about 10·5 per cent. One gram of protein would therefore usually give a solution containing 0·01–0·04 g. tyrosine. To estimate 0·01 g. tyrosine, 0·19 cc. of M/5 bromate solution would be required and an error of 0·05 cc. in the titration would make an error of 25 per cent. in the estimation. It was therefore necessary to ascertain if tyrosine could be brominated by a more dilute solution of sodium bromate. Estimations were therefore made with an N/5 (= M/30) solution of bromate in the same way as described by J. H. Millar, thus:

0·1911 g. tyrosine in 25 cc. hydrochloric acid solution required 21·05 cc. M/30 bromate solution;

i.e. 1 g. tyrosine absorbs 1·75 g. bromine (theoretical 1·765).

The bromination of tyrosine by the M/30 solution of sodium bromate is slower than by the M/5 solution and it appeared that the method might be more convenient if the bromination were effected by adding excess of the sodium bromate solution to the tyrosine solution, allowing the reaction to proceed for 10–15 minutes in a closed flask, adding potassium iodide and titrating the excess of halogen with thiosulphate solution using starch as indicator. 2 g. of tyrosine were dissolved in 500 cc. hydrochloric acid;

50 cc. + 30 cc. M/30 $NaBrO_3$ solution; 7·1 cc. thiosulphate solution; 22·9 cc. bromate required.

50 cc. + 30 cc. M/30 $NaBrO_3$ solution; 7·2 cc. thiosulphate solution; 22·8 cc. bromate required.

i.e. 50 cc. contain 0·207 g. tyrosine.

The absorption of bromine for 1 g. of tyrosine is 1·825 g., a value slightly higher than the theoretical (1·765), but comparable with that found by J. H. Millar (1·808). In subsequent experiments this high figure was not obtained. It seems to have been due to the presence of a rather large excess of bromate solution; the vapour in the flask appeared yellow and loss occurred during the titration.

Small quantities of tyrosine can thus be accurately estimated by making this alteration in the procedure.

(2) *Estimation of Tyrosine in the Presence of Protein and its Products of Hydrolysis.*

It has been shown by A. J. Brown and E. T. Millar that proteins absorb bromine under the conditions employed for the estimation of tyrosine by J. H. Millar's method, but this did not preclude the estimation of tyrosine in the presence of unaltered protein if the amount of bromine absorbed by the protein was deducted from the amount absorbed by the mixture of protein and tyrosine. They showed further that there was no increase in the absorption of bromine by gelatin during its digestion by trypsin. Gelatin does not contain tyrosine or tryptophane, and since it was found that tryptophane did not absorb bromine under the same conditions it was concluded that the increase in the absorption of bromine when edestin was digested by trypsin was entirely due to the liberation of tyrosine. The absorption of bromine due to the separation of tyrosine increased very rapidly and reached a maximum in about one hour. The result gave the tyrosine content of

edestin as 4·06 per cent. which differs very greatly from the value (2·1 per cent.) obtained by the isolation and weighing of tyrosine.

An examination was made for possible sources of error occurring during the estimation of tyrosine by bromination.

Brown and Millar used M/5 sodium bromate solution and 50 cc. of a 1 per cent. solution of edestin.

0·80 cc. of the bromate solution was required for the bromination and from this 0·42 cc. was deducted for the control. The difference of 0·38 cc. gave the tyrosine content.

As shown above an error of 0·05 cc. in the titration, if M/5 sodium bromate solution be used, corresponds to a difference of 25 per cent. in the amount of tyrosine when estimating 0·01 g. of tyrosine, which is equivalent to a variation of 1 per cent. in the tyrosine content of edestin. A proportionate error must be considered when the deduction for the control is made. The error in titration can be reduced by employing M/30 bromate solution. An error of 0·23 cc. in the titration will now correspond to a difference of 10 per cent. in the amount of tyrosine, which is equivalent to a variation of 0·4 per cent. for the tyrosine content of edestin. Dilution of the reagent will thus reduce the error but it is not in itself sufficient to make the method an accurate one.

The amount which has to be deducted for the control is greater than the amount used in the actual estimation; greater accuracy can therefore only be obtained by reducing or eliminating this deduction.

The constituents of a protein which are known to absorb bromine are tyrosine, tryptophane and histidine [Knoop, 1908]. According to Brown and Millar tryptophane does not absorb bromine under the conditions existing during the estimation of tyrosine so that it is most probably the histidine which absorbs the bromine and necessitates the large deduction for the control. Histidine can be removed by precipitation with phosphotungstic acid which leaves the tyrosine (and tryptophane) in solution in the filtrate. Hence if the deduction for the control be due to the presence of histidine, the absorption of bromine by the filtrate should be due solely to the tyrosine contained in the enzyme preparation. A preliminary experiment with gelatin showed that this was the only deduction necessary.

A 2 per cent. solution of trypsin was allowed to digest in the presence of chloroform; at intervals 20 cc. were removed and placed in 50 cc. of 5 per cent. sulphuric acid + 20 cc. of 10 per cent. phosphotungstic acid. To 50 cc. filtrate (= 0·333 g. trypsin) 10 cc. of sodium bromide (2 per cent.) and 10 cc. of the sodium bromate solution (1 cc. = 0·012 g. Br) were added;

after 10–15 minutes 10 cc. sodium iodide (4 per cent. solution) were added and the mixture titrated with sodium thiosulphate solution (1 cc. = 0·00663 g. Br) using starch as indicator[1].

Time	Thiosulphate required	Bromine absorbed
0	15·1 cc.	0·02 g.
4 hours	11·1	0·046
6 ,,	11·8	0·042
24 ,,	12·1	0·040

Taking the absorption after 6 hours, 1 g. of trypsin absorbs 0·126 g. bromine.

A 2 per cent. solution of gelatin in 0·4 per cent. sodium carbonate solution was digested in the presence of chloroform with 0·2 per cent. trypsin. 50 cc. samples were removed at intervals and placed in 50 cc. of 5 per cent. sulphuric acid + 20 cc. of 10 per cent. phosphotungstic acid. 100 cc. of the filtrate (= 0·833 g. gelatin) were treated with 10 cc. sodium bromide solution + 10 cc. sodium bromate solution (1 cc. = 0·012 g. bromine) and after half an hour the mixture was titrated with sodium thiosulphate (1 cc. = 0·00663 g. bromine) after adding sodium iodide and starch as indicator. Each sample contains 0·833 g. trypsin.

Time	Thiosulphate required	Bromine absorbed	Bromine absorbed by 0·0833 g. trypsin	Bromine absorbed by gelatin
0	17·4 cc.	0·0046	0·0025	0·0021
4 hours	16·2	0·0126	0·0104	0·0022
6 ,,	16·25	0·0126	0·0104	0·0022
24 ,,	15·6	—	—	—

The slight absorption by the gelatin (= 0·24 per cent.) is most probably due to the presence of tyrosine; the sample (gold label) gave a distinct reaction with Millon's reagent.

This procedure was then applied to the estimation of the tyrosine in caseinogen.

100 cc. samples of a 1 per cent. caseinogen solution were digested with 5 cc. of a trypsin solution for periods of 1–5 hours and were then precipitated with 25 cc. phosphotungstic acid solution in hydrochloric acid; 50 cc. of the filtrate were used for the titration:

[1] In the presence of phosphotungstic acid it is better to use sodium bromide instead of potassium bromide and sodium iodide instead of potassium iodide as potassium phosphotungstate is precipitated when potassium salts are present and the precipitate interferes with the titration with thiosulphate when starch is used as indicator. Auld and Mosscrop [1913] have maintained that starch and potassium iodide cannot be used when estimating tyrosine in digests of protein by the Millar method. Colourless filtrates are obtained after precipitation with phosphotungstic acid and with the alteration in the procedure the disappearance of the blue colour when the solution is titrated with thiosulphate is quite sharp. No difficulty has been experienced in determining the end point under these conditions.

Time (hours)	M/30 bromate absorbed	Difference
0	2·4 cc.	—
1	3·8	1·4
2	4·1	1·7
3	4·6	2·2
4	4·6	2·2
5	4·55	2·15

The maximum absorption of bromine occurred after 3 hours and then remained constant. The percentage of tyrosine in caseinogen calculated from the above difference is 5·08, a figure which agrees well with those usually given (4·5–5).

The following duplicate experiments with another solution of caseinogen show the reliability of the procedure[1].

	(1)			(2)		
Time (hrs.)	Bromate added	Thiosulphate required	Bromate absorbed	Bromate added	Thiosulphate required	Bromate absorbed
0	5 cc.	9·65 cc.	0·2 cc.	5 cc.	9·55 cc.	0·25 cc.
0·5	,,	7·45	1·3	,,	7·3	1·35
1	,,	5·95	2·05	,,	5·9	2·05
2	,,	4·8	2·6	,,	4·85	2·6
3	,,	4·35	2·85	,,	4·35	2·85

The estimation of tyrosine in the phosphotungstic acid filtrate is thus possible if no other products which absorb bromine are present. Gelatin does not contain tryptophane and cystine and it only contains a small amount of phenylalanine. Cystine and phenylalanine have been found not to absorb bromine, but the behaviour of tryptophane, which according to Brown and Millar does not react with bromine under the conditions adopted by J. H. Millar, probably because of the presence of hydrochloric acid, required further investigation as the method had been modified and as Dr Hopkins had informed us that tryptophane did react with bromine when treated in this way. A quantity of tryptophane was kindly supplied to us by Dr Hopkins for this purpose and the following experiments were carried out:

0·0631 g. tryptophane was dissolved in hydrochloric acid and titrated directly with sodium bromate by Millar's method.

7·3 cc. bromate were required. Bromine absorbed = 0·112 g.

0·0238 g. tryptophane was dissolved in hydrochloric acid: 10 cc. sodium bromide and 10 cc. sodium bromate solution (= 1536 g. Br) were added: after

[1] These experiments were carried out by one of us in conjunction with Mr S. H. Wood and the results were communicated to a meeting of the Physiological Society in March 1907. Continuation of the work was not then possible and no further experiments were made until 1909. The results of these later experiments were communicated to the Biochemical Club in March 1912.

15 minutes titrated with sodium thiosulphate solution (1 cc. = 0·00663 g. Br) after adding sodium iodide and starch: 3·2 cc. were required. Bromine absorbed = 0·1321 g.

0·0200 tryptophane was treated as in the previous experiment: 6·4 cc. thiosulphate were required. Bromine absorbed = 0·1105 g.

0·57 g. tryptophane was dissolved in 1000 cc. water; 100 cc. of this solution were used in each of the following experiments:

(1 cc. $NaBrO_3$ = 0·012 g. Br and 1 cc. thiosulphate = 0·00663 g. Br.)

	HCl	NaBr	$NaBrO_3$	Titrated after	Thiosulphate required	Bromine absorbed
100 cc.	5 cc.	10 cc.	10 cc.	15 mins.	2·05 cc.	0·1064 g.
	10	10	10	,,	1·40	0·1107
	1·5	10	10	,,	2·30	0·1048
	5	10	20	,,	17·95	0·1210
	10	10	20	30 mins.	17·0	0·1270
	5	10	20	,,	16·5	0·1306
	10	10	20	1·5 hrs.	15·05	0·1400

Another series of experiments gave similar results.

Tryptophane thus absorbs bromine under the conditions adopted by Millar; it absorbs a greater amount of bromine under the modified conditions, the absorption increasing with a larger amount of bromate and with the time allowed for the reaction. The absorption corresponds to about 6 atoms of bromine by 1 molecule of tryptophane.

The presence of tryptophane in solution with tyrosine will thus interfere with the estimation of tyrosine.

According to Hopkins and Cole tryptophane is destroyed by prolonged boiling with acids. If its products of decomposition do not absorb bromine the estimation of tyrosine should be possible after acid hydrolysis. Some experiments were therefore made to see if tryptophane still absorbed bromine after boiling with acids:

100 cc. of the above solution of tryptophane were heated for 10 hours with excess of concentrated hydrochloric acid (50 cc.). 10 cc. NaBr + 20 cc. $NaBrO_3$ were added and after 15 minutes titrated with thiosulphate (16·5 cc. required). Bromine absorbed = 0·110 g.

0·058 g. tryptophane was dissolved in 100 cc. water: 10 cc. were titrated directly: 10 cc. after boiling with concentrated hydrochloric acid and 10 cc. after boiling with 25 per cent. sulphuric acid. The bromine absorbed was respectively 0·0361 g., 0·0057 g. and 0·0106 g.

0·0998 g. tryptophane was dissolved in 50 cc. water: 10 cc. were titrated directly and 10 cc. after boiling for 5 hours with hydrochloric acid. The bromine absorptions were respectively 0·1017 g. and 0·074 g.

Absorption of bromine still occurs, but to a less extent, after boiling tryptophane with acid, so that the estimation of tyrosine is not possible after the hydrolysis of protein by acids.

Brown and Millar have shown that the whole of the tyrosine is very rapidly liberated by the action of trypsin and our preliminary experiments with trypsin and caseinogen have confirmed their results. It has been shown by Hopkins and Cole that the liberation of tryptophane does not occur rapidly with moderately active trypsin solutions and that its amount in solution only reaches a maximum after several days. This difference in the rate of liberation of the two substances may therefore permit of the estimation of the tyrosine content of a protein when the bromine absorption is measured at intervals during the digestion and when only those amounts absorbed in the early stages, from 6–24 hours, are taken as a measure of the tyrosine content.

(3) *Absorption of Bromine during the Tryptic Digestion of Proteins.*

Specimens of several animal and vegetable proteins have been procured and examined by the method described above. The vegetable proteins were most kindly sent to us by Prof. Osborne, the specimens in most cases being the same as those in which he and his co-workers had determined the tyrosine content by direct isolation and weighing.

In general, a 1 per cent. solution of the protein in 0·25 per cent. sodium carbonate was digested in the presence of chloroform or carbon tetrachloride with a 0·1 per cent. solution of trypsin. 50 cc. samples were removed immediately and after various intervals of time and then precipitated with 100 cc. of 10 per cent. phosphotungstic acid in 5 per cent. sulphuric acid. 100 cc. of the filtrate were then taken for the estimation. 10 cc. sodium bromide (2 per cent.) and 10 cc. sodium bromate solution were added; after 15 minutes the excess of bromine was displaced by adding 10 cc. of sodium iodide solution (4 per cent.) and the liberated iodine titrated with sodium thiosulphate solution using starch as indicator. The estimations were generally made after periods of 6 hours and 24 hours, since the amount of material at our disposal did not allow of observations at more frequent intervals. These times were chosen as the preliminary experiments with caseinogen showed that the absorption began after about 1 hour and reached a maximum in from 3–5 hours and that another rise sometimes occurred in about 24 hours. The presence of tryptophane in solution was tested for by the bromine reaction in some experiments; it was generally absent in the 6-hour period but was faintly visible in the 24-hour period.

The estimation with silk-fibroin was performed after hydrolysis with 20 per cent. sulphuric acid for 18 hours as trypsin has only a very slight action upon this protein. Silk-fibroin does not contain tryptophane.

Peptone Roche, which is prepared from silk-fibroin by acid hydrolysis, gave only a slight precipitate with phosphotungstic acid; it seems to contain tyrosine or a polypeptide containing tyrosine which reacts with bromine. Like silk-fibroin this protein contains no tryptophane.

It was impossible to estimate the tyrosine content of the alcohol soluble proteins—the gliadins—since they are only digested with extreme slowness by trypsin.

The following are the data:

Trypsin. (Used in experiments with caseinogen, "peptone Roche.")

1 per cent. in 0·25 per cent. Na_2CO_3 solution digested in presence of carbon tetrachloride. 50 cc. samples in 100 cc. phosphotungstic acid solution. 100 cc. filtrate (=0·33 g. trypsin) for estimation. 5 cc. sodium bromate solution (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time (hours)	Thiosulphate required	Br absorbed
0	8·3 cc.	0·0210 g.
6	6·3	0·0345
7	6·65	0·0321
24	6·7	0·0318

Caseinogen. (Hammarsten.)

12·233 g. in 487·27 cc. of 0·25 Na_2CO_3 solution + 122·33 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid solution. 100 cc. filtrate (=0·667 g. caseinogen + 0·0667 g. trypsin). 10 cc. sodium bromate solution (=0·1536 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·00642 for trypsin	Tyrosine content per cent.
0	22·9	—	—	—
5 hours	12·5	0·0696	0·0632	5·35
6 ,,	12·65	0·0695	0·0631	5·34
23 ,,	10·75	0·0814	0·0749	6·35
30 ,,	7·4	0·1036	0·0972	8·23
48 ,,	7·9	0·1005	0·0941	7·98
4 days	7·55	0·1036	0·0972	8·23

"*Peptone Roche.*"

5·735 g. in 514·2 cc. of 0·25 Na_2CO_3 solution + 57·3 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid solution. 100 cc. filtrate (=0·333 g. peptone + 0·0333 g. trypsin) for estimation. 10 cc. sodium bromate (=0·1536 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0021 at 0 hrs. & 0·0032 afterwards for trypsin	Tyrosine content per cent.
0	14·85	0·0998	0·0538	8·79
2·5 hours	14·2	0·0954	0·0582	9·32
3·75 ,,	13·5			
7 ,,	13·1			
9 ,,	13·3	13·4	0·090	0·0636	10·23
24 ,,	13·6			

Silk-fibroin.

4·39 g. were hydrolysed with 20 per cent. H_2SO_4, and the solution made up to 500 cc. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·293 g. fibroin) for estimation. 5 cc. bromate solution (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br). Thiosulphate required=4·1 cc. Br absorbed=0·0493 g. Tyrosine content=9·53 per cent.

Conglutin (Merck) containing 5·2 per cent. moisture.

5·6158 g. in 222·7 cc. Na_2CO_3 solution+56·1 cc. trypsin solution+2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·632 g. dry conglutin) for estimation. 5 cc. bromate (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br)

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br. absorbed after deducting 0·00642 for trypsin	Tyrosine content per cent.
0	11·7	—	—	—
4 hours	8·55	0·0193	0·0129	1·15
6 ,,	8·3	0·0210	0·0146	1·31
24 ,,	5·35	0·0415	0·0351	3·15

Excelsin. (Own preparation.)

2·697 g. in 306 cc. of 0·25 per cent. NaOH+2 cc. $CHCl_3$+2 "holadin" capsules. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·2937 g. excelsin) for estimation.

Time	Total Br absorbed, g.	Br absorbed after deducting 0·002 for holadin	Tyrosine content per cent.
0	0·00287	0·0008	—
5·5 hours	0·0149	0·0129	2·5
24 ,,	0·0258	0·0238	4·6
28 ,,	0·026	0·024	4·61
48 ,,	0·0282	0·026	5·0

Legumin (Osborne) containing 4·07 per cent. moisture.

2·9866 g. in 266·6 cc. Na_2CO_3 solution+30 cc. trypsin solution+2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·03198 g. dry legumin+0·0033 g. trypsin) for estimation. 5 cc. bromate (=0·08375 g. Br). Titrated with thiosulphate (1 cc.=0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·45	0·0012	—	—
6·75 hours	8·15	0·0185	0·0152	2·69
27·5 ,,	7·0	0·0272	0·0239	4·23
3 days	6·0	0·0351	0·0318	5·63

Edestin (Osborne) containing 13·07 per cent. moisture.

4·6052 g. in 182·3 cc. Na_2CO_3 solution+46 cc. trypsin solution+2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·58 g. dry edestin+0·0667 g. trypsin) for estimation. 5 cc. bromate (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·00642 g. for trypsin	Tyrosine content per cent.
0	11·4	—	—	—
4 hours	8·35	0·0207	0·0143	1·4
6 ,,	7·85	0·0241	0·0177	1·73
24 ,,	5·0	0·0432	0·0368	3·6
48 ,,	2·6	0·0593	0·0529	5·17

Vignin (Osborne) containing 7·03 per cent. moisture.

1·8982 g. in 169 cc. Na_2CO_3 solution + 18·9 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·310 g. dry vignin + 0·033 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Titrated with thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·9	—	—	—
6·75 hours	7·95	0·0223	0·0190	3·4
27·5 ,,	6·35	0·0360	0·0327	5·97

Squash Seed Globulin (Osborne) containing 10·02 per cent. moisture.

1·912 g. in 170·1 cc. Na_2CO_3 solution + 19·1 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·3 g. dry protein + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 c.c. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·8	—	—	—
5·75 hours	8·2	0·0205	0·0172	3·24
27 ,,	6·5	0·0339	0·0306	5·8

Amandin (Osborne) containing 10·46 per cent. moisture.

3·3404 g. in 298·6 cc. Na_2CO_3 solution + 33·4 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (0·3 g. dry amandin + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·6	—	—	—
6 hours	8·2	0·01896	0·0157	2·9
24 ,,	6·75	0·0304	0·0271	5·1

Glycinin (Osborne) containing 9·06 per cent. moisture.

1·9166 g. in 170·5 cc. Na_2CO_3 solution + 19·2 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·302 g. dry glycinin + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·4	—	—	—
6 hours	9·3	0·0091	0·00058	1·1
28 ,,	7·7	0·0218	0·0185	3·47

In most cases there was an increase in the bromine absorption between the periods of 6 hours and 24 hours. The exact time at which the increase occurred could not be ascertained since it was impossible to make more frequent determinations owing to the scarcity of material. An increase after a constant period would show the point when all the tyrosine was liberated and that at which the tryptophane became set free. There is a very close

agreement between the figures for the tyrosine content after the 6-hour interval and the figures obtained by isolation and weighing, as is shown by the following table:

Protein	Percentage of tyrosine after 6 hours' digestion	Percentage by weighing
Caseinogen	5·34	4·5
"Peptone Roche"	10·23	—
Silk-fibroin	9·53	9–10·5
Conglutin	1·31	2·1
Legumin	2·69	2·4
Edestin	1·73	2·1
Vignin	3·4	2·3
Squash seed globulin	3·24	3·1
Amandin	2·9	1·1
Glycinin	1·1	1·9
Excelsin	2·5	3·1

The correspondence in the figures in the cases of edestin, glycinin, squash seed globulin, excelsin, legumin and silk-fibroin is very close. The value is 1 per cent. higher for caseinogen, and the value is also higher for amandin and vignin. The result should be slightly higher for excelsin as the material was not dried. The amounts of amandin and glycinin available were very small, so that much stress cannot be placed on these figures.

The method of bromination therefore appears to be of use for the estimation of the tyrosine content of proteins if measurements of the absorption are made at frequent intervals during a tryptic digest of the protein, but it must be used with precautions and the figures carefully criticised.

Summary.

The estimation of small quantities of tyrosine—0·01–0·04 g.—can be effected by J. H. Millar's method of bromination, when a more dilute solution of sodium bromate is used, but it is preferable to modify his procedure by adding excess of the reagent and titrating the non-absorbed halogen with thiosulphate solution, using potassium iodide and starch as indicator. Tyrosine cannot be directly estimated by bromination in the presence of protein and its decomposition products, since histidine and tryptophane also absorb bromine. Histidine can be removed by precipitation with phosphotungstic acid. The absorption of bromine by tryptophane is not completely eliminated after boiling with acids so that tyrosine cannot be estimated by this method in solutions containing the products of acid hydrolysis of proteins which contain tryptophane. Values for the tyrosine

content of proteins, agreeing with those obtained by isolation and weighing, are obtained when the bromine absorption of a tryptic digest is measured after an interval of about 6 hours.

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XXXI. THE SEPARATION OF CYSTINE AND TYROSINE.

By ROBERT HENRY ADERS PLIMMER.

From the Ludwig Mond Research Laboratory for Biological Chemistry, Institute of Physiology, University College, London.

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Cystine and tyrosine resemble each other so closely in their solubility in water, in alkali and in acid, that their separation is not easily effected. Both Mörner [1899, 1901] and Embden [1900] who first isolated cystine from the products of the acid hydrolysis of scleroproteins obtained it mixed with tyrosine. Mörner effected the separation of the two substances by fractional crystallisation from ammonia; Embden by dissolving out the tyrosine with very dilute nitric acid. Friedmann [1902] separated tyrosine from cystine by solution in ammonia and neutralisation with acetic acid; tyrosine crystallised out in the neutral solution and the cystine was precipitated by making the filtrate strongly acid with acetic acid. Cystine is usually prepared from the products of acid hydrolysis of proteins by nearly neutralising with soda and allowing to crystallise. Folin [1910] described the isolation of cystine and tyrosine by neutralising the hydrochloric acid hydrolysis solution to Congo-red with sodium acetate; cystine crystallises out and on diluting the filtrate and allowing it to stand the tyrosine separates out slowly. Cystine thus seems to be the more insoluble in dilute mineral acids and in strong acetic acid.

Neither Mörner's, Embden's, nor Friedmann's method gives a quantitative separation of the two substances; they only permit of the isolation of a portion of the mixture. Folin's method of preparation was devised for obtaining a quantity of cystine. A method for the separation of these two constituents of a protein is therefore required. On account of their similarity the tyrosine isolated from the products of hydrolysis may contain cystine unless this unit has been completely decomposed during the process of

hydrolysis[1]. For this reason the data of the actual amount of cystine in most proteins are scanty or lacking and except in the scleroproteins and in the protein described by Kotake and Knoop [1911] the relative amount of cystine to tyrosine is very small. The estimations of tyrosine which have been made by Plimmer and Miss Eaves [1913] by the method of bromination are not greatly higher than the amounts of tyrosine obtained by direct isolation and weighing. The presence of a small amount of cystine in the tyrosine may explain the slight differences.

Winterstein [1901] described the precipitation of cystine by phosphotungstic acid and Hopkins and Cole [1902] its precipitation by a solution of mercuric sulphate in five per cent. sulphuric acid. Tyrosine is not precipitated by phosphotungstic acid and its mercury compound is soluble in the five per cent. sulphuric acid. Cystine and tyrosine have been found to differ very greatly in their behaviour to absolute alcohol saturated with hydrochloric acid gas. Tyrosine is readily esterified and goes into solution in the acid alcohol; cystine is not readily esterified and is only very slowly dissolved and the portion which goes into solution can be precipitated by adding an equal volume of absolute alcohol. Neither of these differences has hitherto been used for the specific purpose of separating the two compounds and their applicability has therefore been tested.

Experimental.

Tyrosine was prepared by the hydrolysis of caseinogen by trypsin. The products which crystallised out during the digestion and on the concentration of the filtered solution were mixed and purified by repeatedly dissolving in dilute sulphuric acid and exactly neutralising with caustic soda, 14 g. of pure tyrosine being thus obtained from 600 g. of caseinogen. The substance gave no precipitate with phosphotungstic acid showing the absence of diamino-acid (diaminotrihydroxydodecanic acid) and of cystine.

Cystine was prepared from wool and hair by Folin's method; the final product was not perfectly white but it consisted of the typical hexagonal plates.

[1] Cystine is rapidly decomposed by boiling with alkali with the formation of hydrogen sulphide (loosely bound sulphur) and it is also decomposed by prolonged boiling with acid. In preparing cystine from wool or hair the best yield was obtained when the material was boiled with concentrated hydrochloric acid for 3–4 hours; the yield was very poor when the boiling lasted from 5–8 hours. In purifying the cystine by boiling with charcoal in acid solution loss also occurs. If a solution of cystine in dilute hydrochloric acid be boiled for a long time it becomes yellow or yellow-brown in colour.

(1) *Separation by means of Phosphotungstic Acid.*

Cystine and tyrosine were mixed together in different proportions and dissolved in 50 cc. of 5 per cent. sulphuric acid. A 30 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid was added so long as a precipitate was formed. (20 cc. sufficed for the precipitation of 0·5 g. of cystine.) After standing for 12 hours the precipitate was filtered off and washed repeatedly with a 2·5 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid.

The cystine was recovered from the precipitate either by suspending it in water or in water containing acetone as recommended by Wechsler [1911] and adding baryta water until the solution remained permanently alkaline to phenolphthalein, the decomposition being carried out on the water bath. Excess of baryta was carefully avoided so as to prevent decomposition of the cystine. The filtrate from the barium phosphotungstate was acidified with hydrochloric acid, evaporated to a small volume on the water bath, and neutralised with ammonia. The cystine crystallised out and after being left to stand for 1–2 days was filtered off, washed, dried and weighed.

The recovery of the cystine from its phosphotungstate by decomposition with hydrochloric acid and extraction of the reagent with ether (Winterstein) was also attempted; the whole of the reagent was not dissolved by the ether and the recovered cystine was contaminated with phosphotungstic acid. Experiments were not made with amyl alcohol which Jacobs [1912] recommended as a solvent for extracting phosphotungstic acid.

The tyrosine was recovered from the filtrate by adding ammonia to remove the excess of phosphotungstic acid, filtering off the ammonium phosphotungstate, neutralising and evaporating to a small volume. The crystals so obtained were washed with water to remove ammonium sulphate and the tyrosine residue was dried and weighed. This procedure was preferred to the usual method of removing excess of acid with baryta which entails repeated extraction of a bulky precipitate of barium phosphotungstate and sulphate with hot water and the evaporation of a large volume of liquid.

The amounts of cystine and tyrosine taken and recovered were as follows:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	0·5 g.	0·35 g.	0·35 g.
0·5	1·0	0·47	0·6
0·5	1·0	0·45	0·55
1·0	0·5	0·80	0·25
1·0	0·5	0·82	0·4
0·5	—	0·27	—
0·5	—	0·3	—

Neither the cystine nor the tyrosine was completely recovered. The cystine is precipitated practically completely by the phosphotungstic acid; the loss seems to take place in its recovery from the phosphotungstic acid precipitate; the solution must be made slightly alkaline to ensure complete decomposition of the phosphotungstate and some of the cystine is most probably also decomposed by the alkali. Some decomposition may also occur during the evaporation of the acid solution. Except in the last experiment the cystine always contained tyrosine and this accounts for the loss of tyrosine. The cystine was only obtained free from tyrosine in the last experiment in which the precipitate was washed some twenty times by removing it from the filter, stirring up with the washing reagent and again filtering until the washings showed no reaction with Millon's reagent: over 80 per cent. of the tyrosine was then recovered.

2 g. of the cystine recovered from the earlier experiments were found to contain 0·6 g. of tyrosine which was isolated by means of alcohol saturated with hydrochloric acid (as described below).

(2) *Separation by means of Mercuric Sulphate.*

Mixtures of cystine and tyrosine in various proportions were made and dissolved in 5 per cent. sulphuric acid and treated with mercuric sulphate dissolved in 5 per cent. sulphuric acid (Hopkins and Cole's tryptophane reagent) until no further precipitate occurred. The precipitate was filtered off after standing for 12 hours and washed repeatedly with 5 per cent. sulphuric acid by removing from the filter, stirring up with the acid and again filtering until the washings gave no reaction with Millon's reagent.

The cystine was recovered from the precipitate by suspending in water and decomposing with hydrogen sulphide. The filtrate from the mercuric sulphide was evaporated on the water bath to a small volume and then neutralised with ammonia. The cystine crystallised out and was filtered off, washed, dried and weighed. The acid filtrate containing the tyrosine was evaporated on the water bath to about 400 cc. and filtered from the mercuric sulphate which had separated out. A slight excess of ammonia was added and after again filtering the solution was evaporated almost to dryness. The crystals so obtained were filtered off and washed with water until free from ammonium sulphate and the residue of tyrosine was dried and weighed. This procedure was preferred to the removal of the sulphuric acid with baryta which would have necessitated the repeated extraction of the insoluble barium sulphate with boiling water.

As with the previous method the amounts of cystine and tyrosine recovered were far from quantitative as is shown by the following figures:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	—	0·30 g.	—
0·5	—	0·31	—
0·5	0·5 g.	0·26	0·45 g.
0·5	0·5	0·28	0·47
0·5	1·0	0·31	0·75
0·5	1·0	0·15	0·96

The loss of tyrosine was apparently less than that of cystine but the tyrosine was very impure and contained a brown pigment arising from the decomposition of cystine. On further investigation the precipitation of cystine by mercuric sulphate in 5 per cent. sulphuric acid was found to be incomplete, as was shown by an estimation of the nitrogen in an experiment with cystine alone:

1 g. of cystine was dissolved in 100 cc. of 5 per cent. sulphuric acid; 20 cc. were found to contain 0·0224 g. N by Kjeldahl's method. The remaining 80 cc. (= 0·0896 g. N) were precipitated with 21 cc. mercuric sulphate solution. 70 cc. of the filtrate contained 0·0172 g. N.

Hence the amount precipitated was 0·0724 g. or 81 per cent. of the cystine. The amount of cystine recovered from the precipitate by the procedure described above was 0·3 g. instead of 0·6 g. Loss occurs not only in the precipitation but also in evaporating the solution before neutralising with ammonia. Cystine is much more unstable to acid than one is led to expect from the description of its isolation.

(3) *Absolute alcohol saturated with hydrogen chloride.*

Whilst preparing tyrosine ethyl ester from some tyrosine it was observed that complete solution of the material could not be effected and an examination of the insoluble residue showed it to be cystine; 5 g. of the material yielded 0·05 g. cystine and 4 g. yielded 0·2 g. cystine

This difference in the behaviour of cystine and tyrosine suggested a simple method for effecting their separation.

Preliminary experiments were made with pure cystine and pure tyrosine; 0·5 g. cystine was covered with 20 cc. absolute alcohol saturated with hydrogen chloride, warmed on the water bath and allowed to stand for 12 hours. The undissolved substance was filtered off, washed with absolute alcohol, dried and weighed. Yield = 0·39 g. A white precipitate was produced when the wash alcohol came into contact with the filtrate. This

precipitate was filtered off, washed, dried and weighed. Yield = 0·11 g. On dissolving a test portion of each of these quantities in ammonia and allowing to crystallise the typical hexagonal plates characteristic of cystine were formed.

0·5 g. tyrosine was covered with absolute alcohol saturated with hydrogen chloride and warmed on the water bath. Complete solution readily occurred and the tyrosine was converted into its ethyl ester. On adding water and neutralising with sodium carbonate no tyrosine was precipitated, but on acidifying and boiling for 4–5 hours the ester was hydrolysed and on again neutralising with sodium carbonate tyrosine was precipitated. It was filtered off, washed, dried and weighed. Yield = 0·45 g. A further 0·05 g. was obtained on acidifying the filtrate, boiling, and neutralising once more.

A mixture of 0·5 g. of each was then treated in the same way with 30 cc. absolute alcohol saturated with hydrogen chloride. 0·35 g. was insoluble and the alcohol used for washing the residue precipitated an additional 0·1 g. The filtrate after hydrolysis of the tyrosine ester yielded 0·47 g. tyrosine.

In these experiments the cystine was recovered in two fractions, but if an equal volume of absolute alcohol be added to the absolute alcohol saturated with hydrogen chloride before filtering the whole of the cystine can be obtained in one operation. The cystine which goes into solution seems to be cystine hydrochloride, for it dissolves in water. The insoluble portion seems to consist mainly of cystine but a portion of it dissolves if it be washed with water, and is apparently cystine hydrochloride as the cystine is precipitated in hexagonal plates on neutralising with ammonia. There is no evidence that the cystine is converted into its ester. According to Friedmann cystine is not easily converted into its ethyl ester but its methyl ester is more easily obtained (Fischer and Suzuki).

The separation of cystine and tyrosine by this method has been tested by the following experiments:

Taken		Recovered	
Cystine	Tyrosine	Cystine	Tyrosine
0·5 g.	0·0 g.	0·47 g.	0·0 g.
0·5	0·0	0·50	0·0
0·0	0·5	0·0	0·5
0·0	0·5	0·0	0·49
0·5	0·5	0·45	0·47
0·5	1·0	0·49	0·96
1·0	0·5	1·00	0·50

The mixtures were treated with absolute alcohol saturated with hydrogen chloride and warmed on the water bath. An equal volume of alcohol was added and the insoluble cystine filtered off, washed, dried and weighed. The

filtrate was diluted with 2 volumes of water and boiled for 8 hours, water being added when necessary. Tyrosine was precipitated on neutralising; it was filtered off, washed, dried and weighed.

The usefulness of this method is illustrated by the first experiment in which presumably pure tyrosine prepared from wool by Folin's method had been used. The presence of cystine was not observed by microscopic examination and the cystine present was found to be unevenly distributed.

The cystine recovered from the phosphotungstate precipitate above mentioned contained tyrosine; 2 g. contained 1·4 g. cystine and 0·6 g. tyrosine.

A mixture weighing 3 g. was found to contain 1·9 g. cystine; the tyrosine was unfortunately lost. This mixture actually contained 2 g. cystine and 1 g. tyrosine.

Summary.

1. Cystine and tyrosine can be separated from one another by precipitation with phosphotungstic acid. The precipitation of cystine is almost complete, but loss occurs in its recovery from the precipitate. Almost the whole of the tyrosine can be recovered from the filtrate and washings.

2. Cystine and tyrosine can be separated from one another by precipitation with mercuric sulphate in five per cent. sulphuric acid. The cystine is not completely precipitated and the tyrosine which is recovered is impure.

3. Cystine and tyrosine can be completely and quantitatively separated by means of absolute alcohol saturated with hydrogen chloride. The tyrosine is rapidly converted into tyrosine ester and goes into solution. It can be recovered by boiling the solution when diluted with water for eight hours and then neutralising with ammonia. Almost the whole of the cystine is insoluble; the portion which goes into solution (perhaps cystine hydrochloride) is precipitated by adding an equal volume of absolute alcohol. The cystine is not converted into its ethyl ester since on dissolving the insoluble portion in dilute hydrochloric acid and neutralising with ammonia the cystine is precipitated in the typical hexagonal plates.

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XXXII. THE FACTORS CONCERNED IN THE SOLUTION AND PRECIPITATION OF EUGLOBULIN.

By HARRIETTE CHICK.

From the Lister Institute.

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I. Introduction.

There is no reason for regarding euglobulin, the material precipitated from serum by dialysis or by dilution and acidification, as a chemical entity. Michaelis and Rona [1910, 2] are of opinion that it consists of that portion of the total proteins which owes its solution to the dispersing power of the electric charge upon its particles. It is true that the procedure adopted for separating euglobulin is one which renders it iso-electric with the solution; but the conditions determining its dispersion in serum are not so simple as they suggest. Euglobulin, as shown by Hardy [1905], is dissolved by neutral salts, e.g., sodium chloride, in a concentration of 1/10th normal, to form a colloidal solution in which the particles are electrically neutral, a result which I have been able to confirm (see p. 329, below). Serum contains enough salt to produce this effect and unless it is at the same time diluted, the euglobulin it contains cannot be completely precipitated by the addition of the amount of acid necessary to render it iso-electric. The dispersion of euglobulin in serum is due to two entirely distinct causes: (1) Electric charge on the particles owing to the alkalinity of the fluid; (2) Formation of a "soluble" compound with the salt present,

concerning the mechanism of which there are a variety of theories, which are discussed in detail below.

By the process of "heat-denaturation" proteins acquire many of the properties peculiar to euglobulin, as regards the factors conditioning their solution (dispersion) and agglutination (see Hardy [1900], Michaelis and Mostynski [1910] and Chick and Martin [1912]). The results of the last-named, however, afford no support for the opinion expressed by Starke [1900] and Moll [1904] that "albumin" is converted into "globulin" by heating. Abderhalden [1903, 1904] has found evidence of difference in chemical composition between the two sets of proteins which is not obliterated after the former has been heated. Further, the analogy mentioned above is by no means complete; the union of euglobulin with neutral salts to form a dispersion that is without drift in an electric field has no parallel in the case of denaturated serum-proteins.

II. Solution of Euglobulin by Acids and Alkalies, and the Iso-Electric point.

Solution of euglobulin by means of acid or alkali was shown by Hardy [1905] to be associated with the possession by the dispersed particles of an electric charge which was respectively positive or negative in sign.

Michaelis and Rona (1910, 2) found the iso-electric point of euglobulin to be at a concentration of hydrogen-ions equal to 36×10^{-7} normal and to coincide with the point of optimum flocculation for this protein.

The iso-electric point has been re-determined in the present instance and the result of Michaelis and Rona has been confirmed.

The euglobulin was prepared as follows: horse serum was diluted ten times with distilled water and the globulin was precipitated by acidifying with acetic acid (about 3–4 cc. N acetic acid per litre according to the original reaction of the serum). The precipitate was allowed to settle, was centrifuged off, and purified by dissolving in a minimal amount of standard sodium hydroxide solution (according to the amount of the precipitate) and reprecipitating with hydrochloric acid, the precipitate again being separated by centrifuging. This operation was repeated once or twice and the precipitate finally washed with distilled water. A fairly concentrated suspension was made in distilled water from which, by dilution, the material was prepared which was used for the various experiments[1]. The particles of

[1] In some experiments, for example those in Tables IV and V, the euglobulin was prepared from horse-plasma (oxalate) by the method described by Mellanby [p. 339, 1905]. The purification in this case was just as above and no differences were detected between samples prepared by the two methods.

suspensions prepared as above invariably had a slight negative charge and the addition of a little acid was necessary in order to render the particles iso-electric with the solution.

In Table I is shown the degree of dispersion of a sample of euglobulin (0·032 % solution) corresponding with various concentrations of acid and alkali. The charge carried by the particles was at the same time determined

TABLE I.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin, and the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0·032 %.

Exp. No.	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in total volume of 10 cc.	Salt added	Concentration of salt, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on the particles	Degree of agglutination
1	—	0·2	—	—	$0·36 \times 10^{-7}$	−	Faintly opalescent soln.
2	—	0·1	—	—	0·97 ,,	−	Opalescent solution.
3	—	0·0	—	—	32 ,,	−	Agglutinated.
4	·1	—	—	—	748 ,,	−	Agglutinated later.
5	·15	—	—	—	1140 ,,	+	Opalescent solution.
6	·2	—	—	—	1390 ,,	+	Faintly opalescent soln.
7	·5	—	—	—	5080 ,,		Clear solution.
8	·2	—	Na_2SO_4	0·03	0·97 ,,	−	Opalescent solution[1].
9	·5	—	,,	0·05	1590 ,,	−	Opalescent solution[1].

[1] Agglutinated on standing.

by observing their behaviour in an electrical field, using the microscopic method previously employed by Martin and the author [1912, p. 285] in investigating the electrical properties of denaturated proteins. In the 5th column is given the concentration of hydrogen-ions in the various solutions, and the point of optimum agglutination is seen to be at a concentration equal to 32×10^{-7} normal. This figure is in good agreement with that found by Michaelis and Rona.

Confirmation of these values was incidentally obtained in the course of experiments made to elucidate other points. For example, the range of agglutination of another euglobulin suspension, containing 0·6 % protein was determined, after first dissolving in a minimum amount of dilute sodium hydroxide solution, by adding dilute hydrochloric acid to a series of tubes until precipitation occurred and finally dispersion was again obtained. Dispersion corresponded with a concentration of hydrogen-ions equal to $2·2 \times 10^{-7}$

normal on the alkaline side and $62{\cdot}1 \times 10^{-7}$ normal on the acid side. The solutions, of course, all contained a trace of salt.

In case of a third sample of euglobulin, see Table II (containing 0·016 % protein) the limits of agglutination were found to lie between concentrations of hydrogen-ions equal to 5×10^{-7} and 213×10^{-7} normal and precipitation to be rapid at a concentration of 18×10^{-7} normal.

TABLE II.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin and upon the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0·016 %.

Exp. No.	Salt added	Concentration of salt added, in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on particles	Degree of agglutination
1	—	—	0·3	—	—	+	Clear solution.
2	—	—	0·2	—	1280×10^{-7}	+	„ „
3	—	—	0·1	—	213 „	+	Agglutination partial.
4	—	—	0·00	—	18·6 „	−	Agglutination complete.
5	—	—	—	0·1	5·41 „	−	Agglutination partial.
6	—	—	—	0·2	—	−	Dispersed.
7	Na_2SO_4	·02	0·5	—	1010×10^{-7}	−	Agglutination partial, less good than No. 8.
8	„	·03	„	—	137 „	−	Agglutination best, but not quite complete.
9	„	·04	„	—	65·9 „	−	Agglutination less good than No. 8.
10	„	·05	„	—	—	0	Dispersed.
11	„	·07	„	—	—	0	Dispersed.

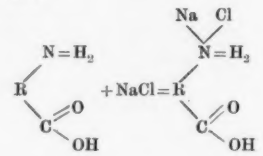
The experiments as to the amount and sign of the charge carried by the euglobulin particles, detailed in Tables I and II, are not calculated to define the iso-electric point with as great accuracy as those of Michaelis and Rona. As a rule the particles, while agglutinating rapidly, retained the negative charge which they originally held and only showed a positive charge when dispersion by acid was already well begun. Speaking generally, however, the point where the charge on the particles changed its sign coincided with the agglutination-zone. On the whole, there is a tendency to retain the negative charge rather than the positive. A distinctly positive charge was first detected on the globulin particles at a concentration of hydrogen-ions

equal to about 200×10^{-7} normal (see Exp. 3, Table II, and Exp. 2, Table VII).

The agglutination and dispersion of euglobulin by acids and alkalies, in confirmation of the results of Michaelis and Rona, is seen to be primarily dependent upon hydrogen-ion concentration, and in this respect a close analogy is presented with heat-denaturated proteins [Michaelis and Rona, 1910, 1; Sörensen and Jürgensen, 1911; Chick and Martin, 1912]. In both cases flocculation takes place when the protein particles are iso-electric with the solution, and, in cases where the reaction of the solution is more acid or alkaline than the iso-electric point, dispersion of the protein is due to the possession of a positive and negative electric charge respectively.

III. Solution of Euglobulin by Neutral Salts.

There has been some difference of opinion as to the mechanism involved in the solution of euglobulin by electrolytes. Hardy [1905] came to the conclusion that salt-solutions of globulin are without drift in an electric field and the particles of the system must be regarded as electrically neutral. He considered [1905, p. 325] "salt-globulin" to be the result of a molecular union of globulin and salt[1] by a process analogous to the formation of amino-acid-salt compounds[2], thus:



A similar theory had already been put forward by Pauli [1899].

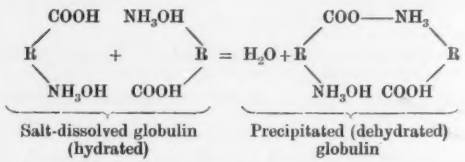
Mellanby [1905], on the other hand, has expressed the opinion that the solution of globulin by electrolytes is the result of activity of the constituent ions. He found that the efficiency of a salt in "dissolving" euglobulin depended on the valency of its ions, and, moreover, that the amount of globulin dissolved by a given concentration of any salt was proportional to the initial concentration of the protein. This curious fact was also noticed by Hardy [1905, p. 310].

A third view is that of Schryver [1910]. He considers (1) that, when precipitated, euglobulin is in a dehydrated condition, the amphoteric molecules having united by twos in salt formation with loss of the elements of

[1] Osborne and Harris [1905] also interpreted the solution of edestin by certain electrolytes as due to the formation of such compounds.

[2] These appear to have been prepared in the crystalline form by Pfeiffer and Modelski [1912].

water and (2) that, when dissolved by electrolytes, the molecules remain separate in their hydrated condition, the aggregation in pairs being prevented by adsorption of the electrolytes on the surface of the molecules, thus



The insolubility of the dehydrated euglobulin Schryver attributes to the increased size of the molecule[1]. There seems to be, however, better experimental evidence in support of the more usual conception that the euglobulin is precipitated in such circumstances as will allow of the aggregation of the protein particles under the influence of surface tension, that is to say, in the absence of other causes tending to keep them dispersed. Such causes are the presence of acid or alkali, in which case the euglobulin particles have been shown to be charged electrically, or the presence of electrolytes in whatever way the latter may act.

Schryver's view is in accord with that of Hardy in so far as the solution of euglobulin by electrolytes is attributed by both to the agency of the salt as a whole rather than to the activity of the constituent ions. His adsorption hypothesis, however, affords no explanation of the comparatively high concentration necessary for solution in case of many salts.

An additional theory, for which some experimental support is here given, is that dispersion of euglobulin by salts may be due to a specific adsorption by the protein particles of one—the more potent—ion of the electrolyte employed, and the acquisition of an electric charge by this means. Such an hypothesis would explain the influence of valency, demonstrated by Mellanby, and be consistent with what is known of the action of neutral salts in dispersing denaturated serum proteins [Chick and Martin, 1912, p. 281], with which euglobulin is in many ways analogous.

In the experiments detailed below, the "solution" of euglobulin by neutral salts was studied, using a series of electrolytes of more varied character, as regards the valency of the constituent ions, than those employed by the above observers. By this means some facts have been discovered which go to show that while, in some instances, solution of euglobulin by

[1] The conclusion of Dabrowski [1912] that in solutions of crystalline egg-albumin containing ammonium sulphate, the colloidal particles are of smaller size than in the case of the dialysed protein, is of interest in this connection.

neutral salts appears to be due to some action of the whole salt, in other cases it is undoubtedly associated with an acquirement of electric charge by the protein particles, the sign of which is determined by the more potent ion of the electrolyte employed.

1. *Experiments to determine whether salt-globulin is electrically charged.*

A series of experiments with a dilute euglobulin suspension (0·016 % protein) and a variety of salts is set forth in Table III. The object was to compare quantitatively the solvent and dispersing power of different ions and to decide whether solution was accompanied by the deposition of an electric charge upon the protein particles. The latter, in this instance, was determined by the microscopic method, using a dark ground and side illumination. The fluids were placed in a specially constructed cell and the behaviour of the particles in an electric field was observed. In using this method with fairly concentrated salt solutions (0·01 normal and upwards) there is some risk of disturbance by gas-bubbles given off at the electrodes, as the result of electrolysis. This has to be very carefully watched. As a rule the difficulty is minimised if the electrodes are previously platinised and the circuit closed only for the short time necessary to make the observation. The electromotive force employed was about 9 volts.

The results of this series of experiments show the relative solvent power of six different salts, viz. the chloride, sulphate and citrate of sodium, the chlorides of barium and calcium and lanthanum nitrate.

It is very clear that the influence of valency in preventing agglutination of euglobulin is far from negligible, multivalent ions being much the more powerful in order of increasing valency. For example, the concentration of lanthanum nitrate and sodium citrate required for the purpose is respectively 1/30th and 1/60th of the necessary concentration of sodium chloride (5th column, Table III).

The electrical condition of the dispersed particles is set out in column 4, and it will be seen that, in four cases, a charge was demonstrated of similar sign to that carried by the more potent ion of the electrolyte employed. In the case of calcium[1] and barium salts, on the other hand, no charge could be detected on the protein particles which remained visible.

The *character* of the dispersion obtained was also different in the case of different salts, and could be grouped according to two types, which I have tentatively called the "electrical" and the "molecular" type of solution.

[1] This was also found to be the case when denaturated serum-proteins were dispersed by calcium salts [Chick and Martin, 1912, Table XIV].

TABLE III.

Solution of euglobulin by electrolytes.

Concentration of protein = 0·016 %.

× × × Complete agglutination, filtrate protein-free.
× × Less complete agglutination, filtrate contained trace protein.
× Partial agglutination.
× – Almost complete dispersion.
– Complete dispersion, clear solution.

Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of the electric charge upon the particles	Concentration of salt, in terms of normality, necessary (*a*) to prevent agglutination	(*b*) to complete dispersion
NaCl	0·01	× × ×		0·03	—
	0·02	× × ×			
	0·03	× ×			
	0·04	× ×			
	0·045	× ×	– *		
	0·05	×	– *		
Na_2SO_4	0·0005	× × ×		0·02	0·05
	0·001	× × ×			
	0·01	× × ×			
	0·02	× ×	– *		
	0·03	×			
	0·04	× –	– *		
	0·05	–			
Na_3Cit	0·0005	×	–	0·0005	0·003
	0·001	×			
	0·002	× –			
	0·003	–			
$CaCl_2$	0·01	× × ×		0·02	—
	0·02	× ×			
	0·03	× ×			
	0·04	× ×			
	0·05	×	0		
$BaCl_2$	0·001	× × ×		0·005	0·05
	0·005	× ×			
	0·008	× ×			
	0·01	× ×	0		
	0·02	×	0		
	0·03	× –			
	0·05	–	0		
$La(NO_3)_3$	0·001	× ×	+	0·001	0·02
	0·003	× ×			
	0·005	× ×			
	0·006	× –	+		
	0·008	× –			
	0·01	× –			
	0·02	–			

* Agglutinated particles.

That with a lanthanum salt or a citrate was exactly analogous to dispersion by acids and alkalies, the globulin suspension, with progressive increase in concentration of the salt, passed through various gradings of opalescent solutions until a clear transparent fluid was finally obtained. At the same time, under the microscope, using a dark ground illumination, innumerable particles could be seen which became smaller and smaller, acquiring at the same time an unmistakably positive or negative charge. Finally the whole field became a uniform grey in colour, until at length no particles could be distinguished.

In the case of the other salts used, there was usually no grading of opalescence, but, as the concentration of salt was increased, a decreasing amount of the original suspension remained undissolved in an otherwise clear fluid. Under the microscope there was a black and white effect, fewer and fewer agglutinated masses remaining visible on the black ground. This type of solution obtained with sodium sulphate and the chlorides of sodium, calcium and barium. In the case of the two latter salts, no charge was discovered on the visible particles; with sodium chloride and sulphate a negative charge was observed on the agglutinated particles which remained visible. It must however be remembered that the sign of the charge in both cases was negative, i.e. similar to that carried by the original globulin suspension.

The microscopic method is clearly unsuitable for studying the electrical condition of salt-globulin in such cases as the above, where "solution" is associated with a degree of dispersion which renders the dispersed particles immediately invisible. A series of corresponding experiments were accordingly made, in which the "U" tube method was employed, the object being to find out whether actual kataphoresis of the "salt-globulin" did or did not take place in an electric field.

This was the method used by Hardy [1905] whose conclusion that "salt-globulin" was without electric charge has already been referred to. In his experiments, however, as was pointed out by Michaelis [1909] the protein solution may not have been adequately protected from the influence of the acid and alkali produced at the electrodes by electrolysis of the salt present.

In order to avoid all complications due to electrolysis five U tubes were arranged in series in the present instance. The "salt globulin" occupied the centre tube, which was connected by a three-way tap on either side for convenience in filling. The other four tubes, two on either side, contained a solution of the same electrolyte in the same concentration as that used to disperse the euglobulin. All five tubes were fastened by brass clips to

a stand, so constructed that their position could be altered until the height of liquid in all the tubes was accurately adjusted to a standard level. Only after this was accomplished were the taps opened, and the euglobulin solution placed in contact with the other tubes. Control experiments showed that under these circumstances there was no transference of liquid from one tube to another. The electrodes were placed in the further arms of the two end tubes and the globulin solution was thus securely protected from the influence of any acid or alkali produced there by electrolysis. Litmus and phenol-phthalein were added to the tubes containing the positive and negative electrodes respectively and in all cases the experiment was discontinued long before there was any danger of acid or alkali reaching the centre tube. The resistance of this arrangement was very great, and, although the electrodes (small strips of platinum foil) were connected with the lighting circuit (200 volts) the current which passed amounted to only 0·0001 to 0·005 ampères according to the nature and concentration of the electrolyte employed. At the close of the experiment, which usually lasted from 5 to 10 hours, the contents of the nearer arms of the U tubes adjacent to the globulin tube were tested for the presence of protein by addition of Esbach's reagent or otherwise.

The results are set out in Table IV below, and, in general, confirm the results of Table III. In the case of calcium chloride, barium chloride and

TABLE IV.

Electrical properties of euglobulin, dispersed by various electrolytes.
(U-*tube method.*)

Protein content = 0·16 %.

Exp. No.	Salt	Concentration, in terms of normality	Electric field, volts.	Current, ampères	Duration of experiment, hours	Sign of electric charge upon protein	Appearance of solution	Sign of electric charge, observed by microscopic method
1	Na_2SO_4	0·05	200	0·0025	8·5	0	Clear solution containing some particles undissolved.	– (agglutinated particles)
2	$CaCl_2$	0·05	,,	0·002	5·5	0	Almost clear solution, containing some particles.	0
3	$BaCl_2$	0·04	,,	0·005	5·0	0	Almost clear solution, faint opalescence.	0
4	$La(NO_3)_3$	0·005	,,	0·0001	5·5	+	Opalescent solution	+
5	Na_3Cit	0·003	,,	0·001	5·0	–	Opalescent solution	
6	,,	0·05	,,	0·0015	9·75	0	Clear solution containing some agglutinated particles.	

sodium sulphate in concentrations of 0·05, 0·04 and 0·05 normal respectively, no migration of the dissolved protein was demonstrated. With the last salt, however, the negative charge carried by the undissolved particles was again shown by the settling which took place less rapidly in the "positive" than in the "negative" arm of the centre U tube, showing that in the latter case the action of gravity was reinforced by motion of the particles towards the positive pole.

Euglobulin dispersed by a small concentration of lanthanum nitrate (= 0·005 normal) displayed a marked positive charge and in the case of sodium citrate[1] a negative charge was shown under similar conditions (concentration = 0·003 normal). If the concentration of sodium citrate was increased to 0·05 normal, however, the dispersed euglobulin showed no migration at all.

The case of sodium citrate is a very interesting one, as the character of the dispersion obtained by employing these two concentrations was also quite different, conforming respectively to the "electrical" and "molecular" types of solution described above (p. 324). With the dilute salt the dispersion is to an opalescent solution and is accompanied by the acquisition of a negative charge; in the stronger solution of citrate, the protein is uncharged and the liquid shows a small precipitate suspended in a clear fluid.

The change from the one type of solution to the other can be seen if a series of solutions be made up containing equal amounts of euglobulin and a concentration of sodium citrate ranging from 0·001 to 0·05 normal. Dispersion is already well marked at 0·001 normal and continues through various grades of diminishing opalescence to a concentration of about 0·003 normal. At a concentration of 0·005 normal the opalescence is greater and at 0·01 normal, a distinct precipitate can be seen. In higher concentrations a gradual clearance takes place, but the solution is now of a different character, less and less of the precipitate remaining undissolved in an otherwise clear solution. In those experiments the protein content was 0·16 %.

Another interesting experiment is the following: The U-tube apparatus was arranged so that in the centre tube was a dispersion of euglobulin in 0·003 N sodium citrate and in the side tubes 0·05 N sodium chloride. As the current passed a precipitation took place in the negative arm of the centre tube, the zone of which continually progressed towards the positive arm. This was followed by a zone of clear solution, also moving in the same

[1] These citrate solutions were carefully prepared to be quite neutral and the hydrogen ion concentration was approximately 10^{-7} normal.

direction. The interface between the advancing chlorine ions and the citrate ions will not be sharply defined, as the former travel more rapidly and tend to over-run and intermix with the latter. A possible explanation is therefore that as both citrate and chlorine ions move towards the positive pole, the latter replace the former in the solution around the protein which is also moving towards the positive electrode, but at a slower rate than either. In low concentration chlorine ions are unable to disperse globulin and precipitation occurs, to be followed again by solution when the concentration of the chlorine ions is sufficiently increased.

The conclusion to be drawn from these experiments is that solution of euglobulin by neutral salts, in case of the more ordinary electrolytes, is due to the formation of some unionised and uncharged compound of salt and globulin—whether by a molecular union (Hardy) or as the result of adsorption (Schryver) there is not, at present, enough experimental evidence to decide. At the same time the phenomenon of dispersion by salts, at any rate in its beginning, is due to an electric charge being deposited on the particles of the euglobulin suspension by the agency of the ions of the electrolyte. The influence of salts upon dispersions of euglobulin by acids and alkalies, dealt with in the next section, is in support of this view.

In the case of the commoner salts, containing only mono- or divalent ions, the electric charges brought into play are not powerful enough to disperse the euglobulin until the concentration is increased to a point where the second ("molecular") type of solution takes place. With such electrolytes the first or "electrical" type of solution as a rule is negligible. It was, however, detected in the case of sodium chloride and sodium sulphate. With sodium citrate, on the other hand, the one type was seen to give place to the other as the concentration of salt was progressively increased.

When euglobulin is denaturated by heat, it loses its characteristic property of forming electrically neutral solutions with electrolytes. For example, after heating a dispersion of euglobulin in 0·05 normal sodium sulphate, in which no kataphoresis of the protein could be demonstrated, the protein particles were found to migrate to the positive pole in an electric field. At the same time an alteration took place in the appearance of the solution—the degree of dispersion was diminished and a thick opalescence was developed. With dispersions in sodium chloride (0·1 normal) and calcium chloride (0·15 normal) almost complete agglutination took place on heating.

2. *Alteration in electrical conductivity during solution of euglobulin by electrolytes.*

It is clear that if a molecular union or an adsorption compound is formed during solution of euglobulin by electrolytes, there must be some diminution in electrical conductivity. Hardy [1905, p. 307] states that a loss of conductivity takes place equal to 1·4 % to 2·4 % in case of solution by magnesium sulphate and sodium chloride respectively.

The results of a special set of experiments, made for the purpose, confirm the result of Hardy.

The same sample of euglobulin was used as for the previous set of experiments, and the mixtures when prepared contained 0·6 % protein. The conductivity was determined:

(1) of the "salt-globulin" solution,

(2) of an equal concentration euglobulin suspension in distilled water.

In the case of (1) and (2) the solutions were allowed to settle or were centrifuged and the conductivity measured in the supernatant liquid.

(3) of an equal concentration salt solution in distilled water.

The conductivity of the distilled water used was found to be negligible in comparison; hence direct comparison was made between (1) and the sum of (2) and (3). All determinations were made at 18°.

The results are set out in Table V and show a loss of conductivity in every case investigated. The salts used were the chlorides of barium and sodium, sodium citrate and lanthanum nitrate. In the case of what I have termed the "electrical" type of dispersion the diminution in conductivity was proportionally greater than with the molecular type; in the former case it occurred with iso-electric euglobulin to the extent of 13·6 % and 11·6 % in case of dispersion by weak (0·004 normal) lanthanum nitrate and sodium citrate respectively, see Exp. 1. With more concentrated salt, 0·05 normal, the loss varied from 1–4 % in the case of the four salts employed.

3. *Relation of the amount dissolved by a salt to the total amount of euglobulin present.*

Notice has already been made of the observation of Mellanby [1905, p. 342] confirmed by Hardy [1905, p. 310] that the amount of euglobulin dissolved by a given concentration of salt is, within certain limits, approximately proportional to the concentration of protein in the original suspension.

In the case of the salt used by Mellanby (sodium chloride in concentration

TABLE V.

Change in electrical conductivity on solution of euglobulin by electrolytes.

Concentration of protein = 0·6 %, temperature 18° C.

Exp. No.	Condition of euglobulin suspension	Salt	Concentration of salt, in terms of normality	Conductivity, in reciprocal ohms: Salt solution	Euglobulin suspension	"Salt-globulin" (found)	"Salt-globulin" (calculated)	Percentage loss in conductivity	Concentration of euglobulin in solution as "Salt-globulin," %
1	Approximately iso-electric	NaCl	0·05	0·1389	0·00050	0·1378	0·1394	1·17	—
		$BaCl_2$	0·05	0·1368	0·00126	0·1368	0·1381	0·99	—
		Na_3Cit	0·004	0·005489	0·00046	0·005331	0·005949	11·6	—
		,,	0·05	0·05289	0·00046	0·05236	0·05335	1·90	—
		$La(NO_3)_3$	0·004	0·006785	0·000345	0·006277	0·007130	13·6	—
		,,	0·05	0·06708	,,	0·06536	0·006742	3·14	—
2	Alkaline	Na_3Cit	0·004	0·005562	0·000569	0·005578	0·006131	9·93	0·28
		,,	0·05	0·05357	,,	0·05337	0·05414	1·45	0·41
		$La(NO_3)_3$	0·004	0·006845	,,	0·006482	0·007414	14·4	0·34
		,,	0·05	0·06681	,,	0·06511	0·06738	3·5	0·51
3	Acid	Na_3Cit	0·004	0·005541	0·002145	0·006907	0·007686	11·3	0·07
		,,	0·05	0·05359	,,	0·05415	0·05573	2·93	0·42
		$La(NO_3)_3$	0·004	0·006850	,,	0·008264	0·008995	8·85	0·45
		,,	0·05	0·06794	,,	0·06717	0·07008	4·33	0·53

from 0·04 to 0·09 normal) solution is of the "molecular" type. On the assumption that this solution is the result of a molecular union between salt and protein (Hardy) it is difficult to explain the existence of the above relationship. On the other hand, if Schryver's view of the salt-solution of euglobulin be accepted in one respect, that is to say if we consider the second or "molecular" type of globulin to be the result of "adsorption" of the salt as a whole, the amount of globulin dispersed by a given concentration of salt might be approximately proportional to the extent of adsorbing surface i.e. to the concentration of euglobulin[1].

It was also possible, however, that solution-rate might be conditioned by the *size* of the euglobulin particles (of which there would be every variety in such a suspension as that used by Mellanby) and that in the time of experiment, final equilibrium had not been maintained but only the particles of small size and comparatively large surface had been successfully attacked.

[1] The experimental support for this view is based upon the fact that the globulin-dissolving capacity and surface-tension (against air) of series of solutions of similar salts, were found to be inversely related to one another, which is the result that should obtain, in accordance with the Willard Gibbs hypothesis. The application of surface tension measurements of air against solution to the case of the protein against solution is not, however, without risk of error.

Some experiments were made to examine this theory, using equal volumes of a 3% suspension of globulin and 1/10th normal sodium chloride solution. It was found, however, that almost perfect equilibrium was attained within a few minutes after mixing. Experiments were also made, using in the one case finely divided euglobulin and in the other a similar suspension of globulin previously aggregated by freezing; the amount of protein dissolved in the second case by 0·05 normal sodium chloride, under similar conditions, was about 20% less than in the former. A phenomenon of this magnitude is, in itself, inadequate to explain the relationship observed by Mellanby and Hardy; it may, however, be a contributing factor.

The facts would appear to be best met by some such conception as the following: Solution of euglobulin by such electrolytes as sodium chloride or sodium sulphate, is due to the formation of a "soluble" compound of the globulin and the salt, which is electrically neutral, and to prevent the dissociation of which a large excess of the salt is necessary. Under such circumstances the final equilibrium, i.e. the relative proportion of "dissolved" and "undissolved" globulin, will largely depend upon the concentration of the salt employed and show an approximate constancy if the latter is maintained constant. In other words, the amount of dissolved euglobulin will be roughly proportional to the original concentration (Mellanby).

IV. The effect of Neutral Salts upon Acid and Alkaline dispersions of Euglobulin.

The influence of salts in causing precipitation of euglobulin previously dissolved in acid and alkali is analogous to the corresponding action with denaturated serum proteins.

Hardy [1905, p. 317] has drawn attention to the fact that the precipitating action[1] of a salt upon "acid or alkali-globulin" is due to one only of its ions, viz.: that which carries a charge opposite in sign to that carried by the protein, and that the higher the valency of this ion, the greater is the power of the salt.

These results have been confirmed by me and I have also been able to show that, if the concentration of salt is further increased, dispersion will again occur, the particles now taking a charge whose sign is determined by that of the more potent ion of the electrolyte employed. Thus the addition of an appropriate electrolyte to an alkaline or acid solution of euglobulin

[1] Mellanby [1905] has dealt with the precipitation of globulin from its solution in electrolytes. This is a different phenomenon and a high concentration of salt is required (salting out).

may, after first precipitating the protein, cause the particles to disperse again with a charge opposite in sign to that originally carried.

For example, an alkaline dispersion of euglobulin containing 0·016 % protein, with the particles negatively charged, was precipitated by the addition of lanthanum nitrate to a concentration of 0·001 normal; at a concentration of 0·008 to 0·01 normal, dispersion again took place, the particles now being positively charged. In a similar experiment with sodium chloride the precipitation took place at a concentration of salt equal to 0·02 normal; at 0·05 normal the globulin was again dispersed, this time bearing a negative charge.

These results present a close analogy with what takes place in case of denaturated serum-proteins, where the degree of dispersion in acid and alkaline solution is also greatly influenced by the presence of neutral salts, and in two ways. In the first place the reaction of protein-containing solutions, whether denaturated or not, becomes altered on addition of neutral salts [Chick and Martin, 1911, p. 21; 1912, p. 280], being shifted in the direction of the neutral point. In acid solution, the concentration of hydrogen ions is lowered and the concentration of hydroxyl ions lessened if the solution be alkaline; the effect is related to the valency of the anion and kation respectively of the electrolytes in the two cases. In the second place, the electric charge carried by the protein particles is modified and may be lessened or even changed in sign by the addition of electrolytes if of opposite sense to that carried by the more potent ion of the electrolyte added.

The effect of the above-mentioned salts in modifying the reaction[1] of solutions containing protein has also been demonstrated in the case of euglobulin (see Tables I, VI and VII). It was therefore necessary to determine in how far the precipitating effect of salts was due to this effect. It is evident that solutions either too acid or too alkaline for precipitation of euglobulin might be adjusted to the iso-electric reaction by the addition of an appropriate electrolyte, and indeed this frequently occurred. For example (Experiment 12, Table VI), addition of sodium citrate to a concentration of 0·002 N, caused precipitation of an acid dispersion of euglobulin (0·016 % protein) at the same time reducing the concentration of hydrogen ions to a point very near the iso-electric point for this protein. In some cases the change of reaction extended to the other side of the iso-electric point, e.g. Table VI, Experiment 13; in this case the observed change of sign in electric charge taking place simultaneously with dispersion by sodium citrate, could be explained on the ground of change of reaction alone. By comparison of

[1] The phenomenon in absence of protein is perceptible but negligible.

Experiment 7 with 12 and 13, Table VI, this change in reaction is seen to be increased proportionally with the degree of valency possessed by the ions of the salt employed.

TABLE VI.

The effect of sodium sulphate and citrate upon an acid dispersion of euglobulin (0·016 %).

Exp. No.	Salt added	Concentration of salt in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0·0		−	Partial agglutination.
2		—	0·075		−	Agglutinated completely.
3		—	0·1		−	,, ,,
4		—	0·2	$10^{-4.10}(810\times10^{-7})$	+	Dispersed, clear solution.
5		—	0·3		+	,, ,, ,,
6		—	0·7		+	,, ,, ,,
7	Na_2SO_4	0·03	0·5	$10^{-4.45}(357\times10^{-7})$	−	Agglutinated.
8		0·04	,,		−	Partial agglutination.
9		0·05	,,		−	Dispersed.
10		0·07	,,		0	,,
11	Na_3Cit	0·001	0·7		+	Dispersed.
12		0·002	,,	$10^{-5.41}(39\times10^{-7})$	−	Almost complete agglutination.
13		0·003	,,	$10^{-6.04}(9{\cdot}2\times10^{-7})$	··	Dispersed.
14		0·004	,,		−	Dispersed, faintly opalescent solution.

In many cases, however, acid and alkaline solutions of euglobulin, with their positively and negatively charged particles respectively, were first precipitated and afterwards dispersed, the particles bearing an electric charge of changed sign in solutions whose reaction still remained more acid and more alkaline respectively, than the iso-electric point[1]. A good example of this is seen in Table I, Experiment 9, where addition of 0·05 N sodium sulphate to an acid dispersion of euglobulin (0·032 % protein) caused the particles to be dispersed and to carry a negative charge in a solution where the hydrogen ion concentration, equal to 1590×10^{-7} normal, was far on the acid side of the iso-electric point. Experiment 7 of Table II is another instance of the same phenomenon[1].

With an alkaline suspension of euglobulin a corresponding series of results was obtained with lanthanum nitrate. The results are set out in

[1] In higher concentration of sodium sulphate, the dispersed globulin appears to be electrically neutral, see Exp. 10, Table VI.

Table VII. A preliminary set of experiments (1 to 8), in absence of electrolytes, showed a positive charge to be acquired by the protein particles at a concentration of hydrogen ions equal to 204×10^{-7} normal. In the alkaline suspension used, the concentration of hydrogen ions was equal to $0{\cdot}0013 \times 10^{-7}$ normal. In presence of 0·006 normal lanthanum nitrate the

TABLE VII.

Effect of lanthanum nitrate upon an alkaline dispersion of euglobulin.

Protein = 0·016 %.

Exp. No.	Salt added	Concentration of salt, in terms of normality	Cc. N/100 HCl (or equivalent) added in a total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in a total volume of 10 cc.	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0·2	—			Dispersed, clear solution.
2		—	0·1	—	$\{10^{-4.69}\ (204 \times 10^{-7})$	+	Dispersed, faintly opalescent solution.
3		—	0·05	—	$\{10^{-7.00}\ (1{\cdot}05 \times 10^{-7})$	−	Agglutinated.
4		—	0	—		−	Dispersed, opalescent sol.
5		—	—	0·5			,, ,, ,,
6		—	—	0·1			Dispersed, clear solution.
7		—	—	0·2			,, ,, ,,
8		—	—	0·5	$\{10^{-9.88}\ (0{\cdot}0013 \times 10^{-7})$		,, ,, ,,
9	$La(NO_3)_3$	0·0005	—	0·5			Dispersed, opalescent sol.
10		0·001	—	,,			Agglutinated.
11		0·002	—	,,		+	Agglutinated almost completely.
12		0·004	—	,,		+	,, ,, ,,
13		0·005	—	,,		+	Agglutination not complete.
14		0·006	—	,,	$\{10^{-7.63}\ (0{\cdot}24 \times 10^{-7})$	+	,, ,, ,,
15		0·008	—	,,			Dispersed partly.
16		0·01	—	,,			,, ,, ,,
17		0·02	—	,,			,, ,, ,,

alkalinity was reduced almost to the neutral point. At the same time the charge on the particles was found to be positive in a solution whose reaction was on the alkaline side of the iso-electric point, and where, in the absence of any electrolyte, the particles would be negatively charged.

In these instances the effect of electrolytes recalls the exactly similar set of phenomena obtaining in the case of denaturated serum-proteins mentioned

above, and also the analogous influence of salts which has been observed in case of inorganic colloidal solutions [Burton, 1909]. In certain of the latter there has been demonstrated a selective adsorption of the ion bearing a charge opposite in sign to that of the charged colloidal particles [Linder and Picton, 1895; Whitney and Ober, 1902; Freundlich, 1910] and this explanation may be extended to the case of proteins.

In this connection, certain of the conductivity determinations in Table V, i.e., those of Experiments 2 and 3, are of special interest. These experiments were made with suspensions of euglobulin which were slightly on the acid and alkaline side, respectively, of the iso-electric point. The effect of adding electrolytes containing trivalent positive ($La(NO_3)_3$) and negative (Na_3Cit) ions was carefully studied. The loss of conductivity was found to be greater for sodium citrate if the globulin suspension were originally acid (i.e. charge on the protein particles positive) and greater for lanthanum nitrate if the globulin suspension were alkaline. At the same time, the degree of dispersion was measured by the content of protein in the supernatant fluid after centrifuging (see last column), and was found to be less. In all cases equivalent solutions of the salts were compared and the effects were more marked in the experiments with less concentrated salt (0·004 normal), in which case dispersion has been shown to be of the "electrical" type.

Analogy with euglobulin presented by caseinogen and other proteins.

Caseinogen has been found to show close analogy with euglobulin as regards the effects of electrolytes upon its solutions in either acid or alkali.

Michaelis and Rona [1910, 2] drew attention to the fact that among the naturally occurring proteins whose solution was accompanied by the acquisition of electric properties, in addition to globulin, were caseinogen, gliadin, and edestin, and they determined the iso-electric point in each case. The result was especially interesting in the case of caseinogen, where the particles were found to be iso-electric with the solution, and to be precipitated when the concentration of hydrogen ions was $1{\cdot}8 \times 10^{-5}$ normal, a degree of acidity far beyond that determined for euglobulin or for the heat-denaturated proteins of serum.

The following experiments, set out in Table VIII, show the analogy with euglobulin to be closely maintained in respect also of the action of electrolytes. For example, in an opalescent solution (0·05 %) in weak hydrochloric acid, in which the protein particles were yet visible under the microscope using a high power and dark ground illumination, the caseinogen was found

to be positively electrified. This solution was readily precipitated by a minute concentration of sodium sulphate (equal to 0·0005 normal) or sodium citrate (0·00005 normal); with increased concentration of either salt (0·015 and 0·001 normal, respectively) the protein particles were again dispersed and found to carry a negative charge in both cases, see Table VIII, Experiment A.

TABLE VIII.

Influence of electrolytes upon agglutination of caseinogen (Merck's pure casein).

(A) Dispersed with a little HCl. (B) Dispersed with a little NaOH.

× × × Complete agglutination. × Partial agglutination.
× × Almost complete agglutination. × – Almost complete dispersion.
– Complete dispersion.

Exp. No.	Concentration of protein, %	Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of electric charge carried by the particles
(A)	0·05	0	0·00	–	+
	,,	Na_2SO_4	0·0005	× × ×	
			0·001	× ×	
			0·005	× ×	
			0·01	× –	
			0·015	–	–
			0·02	–	
	,,	Na_3Cit	0·00001	× ×	
			0·00005	× × ×	
			0·0001	× ×	
			0·0005	×	
			0·001	–	–
			0·002	–	
(B)	0·03	—	0·00	–	–
	,,	$CaCl_2$	0·01	–	
			0·02	× –	–
			0·05	×	–
			0·09	×	0
			0·10	×	
			0·15	×	
			0·2	× –	0
			0·5	–	
	,,	$La(NO_3)_3$	0·00002	–	
			0·00005	×	–
			0·00008	×	
			0·0001	× ×	
			0·0002	× × ×	
			0·0005	× –	
			0·001	–	+

Experiment (B) with an alkaline dispersion of caseinogen 0·03 %, showed an exactly analogous set of phenomena with lanthanum nitrate.

With calcium salts the action appears to be different in character. In the first place no complete precipitation takes place; the size of the particles is increased, but no complete agglutination occurs although the solution becomes turbid. The particles, when dispersed again by increased concentration of the salt (0·2 to 0·5 normal) do not appear to carry any electric charge. In this respect also the analogy with both denaturated serum-proteins and with euglobulin is maintained.

V. Summary.

1. The iso-electric point for euglobulin has been re-determined and found to coincide with the point of most rapid agglutination, viz.: at a hydrogen ion concentration of about 3×10^{-6} normal, a figure which agrees with that obtained by Michaelis and Rona [1910, 2].

2. The solution or dispersion of euglobulin by electrolytes is shown to be much influenced by the nature (especially as regards valency) of the constituent ions and to be of two general types:

(*a*) "electrical" type of solution in which the euglobulin dispersion is accompanied by the acquisition of an electric charge by the protein particles, the sign of which is similar to that of the more potent ion of the electrolyte employed.

(*b*) "molecular" type of solution, in which the dissolved euglobulin is electrically neutral.

In type (*a*) the dispersion is considered to result from a specific adsorption of the ion possessing the higher valency, in (*b*) from a molecular union with (Hardy) or adsorption of (Schryver) the salt as a whole. Both types of solution are accompanied by loss of electrical conductivity in the liquid.

The "electrical" type of solution is well seen in case of dispersion by such salts as sodium citrate and lanthanum nitrate in low concentration; in case of the more ordinary salts, containing mono- or divalent ions only, the electric forces concerned are not powerful enough to disperse globulin until the concentration is raised to a point where "molecular" solution takes place. In the case of sodium citrate, the "electrical" type of solution was found to change to the "molecular" type as the concentration of the salt was increased.

3. Euglobulin, when denaturated by heat, no longer possesses the property of forming the "molecular" type of solution with electrolytes. On

heating the latter, in some cases the degree of dispersion is merely diminished, and the protein particles acquire an electric charge, whose sign is determined by the more potent ion of the electrolyte employed; in other cases agglutination takes place.

4. The reaction of acid and alkaline solutions of euglobulin is greatly influenced by the addition of electrolytes, the hydrogen and hydroxyl ion concentration being reduced respectively. In case of the former the effect is much increased with rising valency of the anion and in alkaline solution the result is determined by the valency of the kation.

5. The influence of electrolytes in causing precipitation of globulin dissolved in acid and alkali may, in some instances, be adequately explained by the alteration in reaction, described under 4; in this way solutions too acid or too alkaline for agglutination of the globulin may be adjusted to the iso-electric point by the addition of an appropriate electrolyte.

Precipitation by electrolytes may, however, also take place in solutions whose reaction is still far removed from that of the iso-electric point. In these instances it is attributed to neutralisation of the electric charge originally carried by the protein particles by means of a specific adsorption of the oppositely charged ion of the electrolyte; the effect is related to valency.

6. In the properties regarding solution and precipitation detailed under 1, 4 and 5, euglobulin, in common with caseinogen, and the vegetable globulins presents a very interesting analogy with heat-denaturated proteins. Euglobulin differs from heat-denaturated protein in its capacity to form solutions with electrolytes in which the protein particles are electrically neutral.

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